(54) Title: METHODS AND COMPOSITIONS USEFUL IN PREVENTING EQUINE INFLUENZA

(57) Abstract

Recombinant vaccines for immunizing horses against equine influenza virus (EIV). The DNA sequences encoding the hemagglutinin (HA) and neuraminidase (NA) glycoproteins from the two strains of EIV currently infective in horses are used to construct vaccinia carried vaccines, to design synthetic peptides for primer and booster administration, and to permit recombinant synthesis of HA and/or NA protein based vaccines. These DNA sequences also provide probes useful for preparing similar vaccines from fresh isolates of new strains generated by genetic drift.

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METHODS AND COMPOSITIONS USEFUL IN PREVENTING EQUINE INFLUENZA

Technical Field

The invention relates to immunizing horses against infection by influenza virus. More particularly, the invention relates to use of vaccinia-carried immunogens and synthetic peptide vaccines useful for this purpose.

Background Art

Equine influenza is a highly contagious respiratory infection engendered in horses by equine influenza virus (EIV). While the disease has low mortality, the economic impact is often great due to deterioration of the subject's performance (Mumford, J.A., et al, Equine Vet J (1980) 12:3-9). Current immunization techniques use inactivated or killed EIV. These procedures are characterized by undesirable side effects, and the immunity conferred appears in some cases to last for no more than three or four months. In short, there is no entirely satisfactory vaccine available to prevent the spread of this disease.
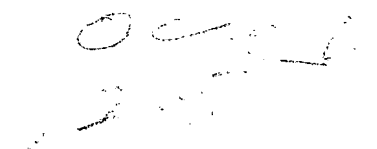
Two different serotypes, which are members of the influenza A myxovirus group, have been identified as causative agents of this disease: A/equine/Prague/1/56 (Sovinova, O., et al, Acta Virol (English ed.) (1958) 2:52-61) (designated herein EIV-A1) and A/equine/Miami/1/63 (Waddell, G.H., et al, J Am Vet Med Assoc (1963) 143:587-590) (designated herein EIV-A2). These strains share the characteristics associated with influenza viruses in general, including those viruses responsible for human influenza. Most importantly, the immunological characteristics of influenza virions

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appear to reside primarily in two virally encoded
glycoproteins, the hemagglutinin protein (HA) and the
neuraminidase protein (NA), both of which are embedded
in the membranous envelope which comprises the outer
5   layer of the virus. These proteins attach themselves to
the outer membrane of infected cells. Twelve subtypes
of HA (H1-H12) and nine subtypes of NA (N1-N9) have been
defined using serological cross-reactivity. Thus, all
influenza virus isolates carry a parenthetical
10   description (HxNy) corresponding to the subclasses
carried. The two equine strains are designated H7N7
(for EIV-A1) and H3N8 (for EIV-A2). (The designation H3
now is used to include both human H3 and former equine
HEq2, although these are not identical proteins. H7 is
15   now used to include both avian Hav7 and former HEq1;
these, too, are not identical. N7 is former NEq1 and N8
is former NEq2. (Melnick, J., _Prog Med Virol_ (1980)
_26_:214-232).)
       Both HA and NA are involved in the disease
20   process. HA functions in attachment to host membrane
and penetration into the host's cells; immunization in
subject experimental animals with HA alone elicits
neutralizing antibodies and protection from the
disease. NA functions in cell-to-cell transmission, and
25   antisera raised against NA attenuate the disease and
decrease the spread of the disease from cell to cell.
(Schulman, J.L., in _The Influenza Viruses and Influenza_
(1975), E.D. Kilbourne, ed, New York/London: Academic
Press, pp. 373-393.)
30   Human influenza virus and the design of
vaccines to protect against it have received
considerable attention. Synthetic peptides designed to
correspond to a putative antigenic epitope on human HA
glycoproteins have been attached to carrier proteins and

used to immunize mice against infection with the human
influenza virus of the same strain (U.S. Patent
4,474,757). The peptides synthesized were apparently
designed based on the sequence of fragments generated by
5   CNBr digestion of the hemagglutinin protein. Synthetic
peptide-carrier vaccines have also been suggested as a
general approach to protection against viral infection
(Brown, F., Ann Rev Microbiol (1984) 3:221-235).

       An alternative approach to vaccine compositions
10   which has been suggested recently utilizes the vaccinia
virus as a carrier. Mackett, M., et al, J Virol (1984)
49:857-864 describes this general method. Briefly,
vaccinia is a large (187 kb) double-stranded DNA virus
which replicates in the cytoplasm of infected cells. It
15   is noninfectious when deproteinized, as it carries its
own enzymes for transcription and cannot utilize the
machinery of the host cell for this purpose. Vaccinia
virus per se was used as the original smallpox vaccine,
and is highly desirable as a vaccine carrier because of
20   its low cost and ease of propagation. Freeze-dried
vaccinia virus used against smallpox could be mass
produced for as little as two cents per dose, while
other subunit vaccines, for example, those against
hepatitis B, cost approximately $100 per course of
25   immunization. There are other advantages as well.
Vaccinia stimulates both the humoral antibody and
cell-mediated immunity systems of the subject. The
freeze-dried vaccine is stable without refrigeration and
is generally potent after a single inoculation. It is
30   also easy to administer under nonsterile field
conditions (Smith, G.L., et al, Biotechniques (1984)
Nov/Dec:306-312). Because of these advantages, vaccinia
has been used as a carrier for antigenic proteins of
hepatitis B and herpes simplex (Paoletti, E., et al,

Proc Natl Acad Sci (USA) (1984) 81:193-197), rabies
(Wiktor, T.J., et al, Proc Natl Acad Sci (USA) (1984)
81:7194-7198), and human influenza hemagglutinin
(Panicali, D., et al, Proc Natl Acad Sci (1983)
5   80:5364-5368; Smith, G.L., et al, Proc Natl Acad Sci
(1983), 80:7155-7159).

        One reason these techniques have not been
extensible to equine influenza is the absence of
sufficient information on the HA and NA proteins of
10   these viruses. This deficiency is remedied by the
present invention, which provides complete genomic and
amino acid sequences for the four relevant surface
proteins characterizing EIV. In addition, the invention
provides properly designed vectors and peptides based on
15   these sequences useful in vaccine compositions.

Disclosure of the Invention
        The invention provides complete genetic
sequences encoding the four glycoproteins which
characterize the two identified serotypes of EIV. The
20   invention further provides vaccine compositions which
are grounded in this genetic information. One class of
vaccine compositions comprises modified vaccinia
engineered to express the HA and NA surface
glycoproteins of the equine infective agents. Another
25   class of compositions comprises synthetic peptide
sequences designed to correspond to the antigenic
determinants of the HA proteins. These synthetic
peptides can be size enhanced, such as by linkage to
carrier proteins, to engender an immune response. Still
30   another class comprises the HA or NA proteins per se,
which are preferably produced recombinantly in host
cells which permit correct post-translational
processing. The vaccine compositions of the invention

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may be used in appropriate protocols for protection of
horses against the disease, either in independent
vaccine regimes, or as adjuncts to more traditional
immunizations using killed virus vaccines. A variety of
5   protocols are workable, and several optimal methods
based on these protocols form a part of the invention.
      The genetic information contained in the cloned
cDNAs representing the EIV glycoproteins is useful in
two other ways--to construct diagnostic probes for
10   detection of the disease and to construct probes to
obtain new cDNAs associated with mutated forms of the
virus.
      Thus, in one aspect the invention is directed
to recombinant DNA comprising the sequences encoding
15   equine H7, equine H3, equine N7, and equine N8. These
recombinant sequences or substantial portions thereof
are useful as diagnostic probes for detecting the
presence of the disease and as probes to retrieve
corresponding cDNA in mutated forms of the virus. These
20   sequences or immunologically effective portions thereof
are useful in preparing vaccines against EIV which are
constructed by inserting them into nonessential portions
of the vaccinia virus genome. Accordingly, in another
aspect, the invention relates to the resulting
25   vaccinia-borne compositions. In still another aspect,
the invention relates to specific synthetic peptide
sequences useful in preparing vaccines effective against
EIV. The latter vaccines are prepared either by using
these peptides per se or by polymerizing these synthetic
30   sequences or attaching them to carrier proteins to
provide enhanced size for immunogenicity. The resulting
vaccines also form an aspect of the invention. In still
another aspect, the invention relates to vaccines
comprised of the HA and/or NA proteins per se. In

addition, the invention includes methods to immunize
horses against EIV infection by administration of the
foregoing compositions, and to prepare vaccines against
mutated forms of the virus.

Brief Description of the Drawings
        Figures 1-4 show the coding sequences and
deduced amino-acid sequences representing the genes and
proteins corresponding to H7 (EIV-Al), H3 (EIV-A2), N7
(EIV-Al), and N8 (EIV-A2), respectively.
        Figure 5 shows the general method to prepare
recombinant vaccinia capable of expressing the EIV
derived genes.
        Figure 6 shows the construction of vectors for
preparing recombinant vaccinia bearing the coding
sequences of Figures 1-4.

Modes of Carrying Out the Invention
A.   Definitions and Description of Surface Viral Proteins
        Hemagglutinin (HA) in general is a glycoprotein
of molecular weight approximately 77 kD, and can be
cleaved by protease action and subsequent reduction of
the single disulfide bond into an amino terminal HA1 50
kD portion and the carboxy terminal HA2 27 kD portion.
Complete nucleotide sequences have been determined for
at least four avian and human subtypes: H1, H2, H3, and
H7. The primary amino acid sequences of all these HA
proteins contain highly conserved cysteine, proline, and
glycine residues and glycosylation sites.
        Crystallographic studies show that human H3
contains a globular head which extends away from the
cellular membrane composed almost entirely of residues
of HA1, and an anchoring stalk portion which is
comprised of mostly HA2 (Wilson, et al. Nature (1981)

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289:366-373). This is believed to be representative for
all HA molecules. Four major antigenic sites, all
located in the HA1 globular portion, have been
identified from less conserved sequences in
5   immunological variants (Wiley, et al, Nature (1981)
289:373-378). As will be seen below, the amino acid
sequence of the equine HA polypeptide differs from those
previously determined for the forms infective in humans,
particularly in the area of the antigenic sites. In
10   addition, HA peptides in general appear to be subject to
antigenic drift with time, although the HA protein from
EIV-A1 appears relatively stable.

Neuraminidase (NA) is an approximately 56 kD
glycoprotein which occurs as a tetramer on the surface
15   of infected cells. Unlike HA, the membrane anchor is at
the amino terminus, and the reactive antigenic sites are
predominantly in the carboxy terminal two-thirds of the
peptide. Comparison of amino acid sequences from
representative N1 and N2 human infective serotypes show
20   extensive homology in the antigenic portion of the
molecule, but little in the stalk portion. A similar
relationship exists between the EIV sequences shown
below.

The equine HA and NA DNA and polypeptide
25   sequences described below and in the figures are
representative of their respective equine subtypes. It
is recognized that minor mutational changes may occur
without destroying the functionality of the DNA
sequences in their function as vaccines per se, or that
30   of the proteins generated by them. It is not intended
that equine H3, equine H7, equine N7, and equine N8 be
limited to the precise nucleotide and amino acid
sequences shown. To fall within the designations, a
particular sequence must only be functionally

substantially identical to those depicted in the
figures.

One obvious source of variation which has
minimal effect on functionality as a coding sequence
5   resides in the redundancy of the genetic code. Of
course, the designations are intended to include
variations in the sequence which result from this codon
redundancy. In addition, minor modifications of the
amino acid sequence, and thus the coding sequence, may
10   be permitted which still result in proteins capable of
the immunological effect desired. Such changes may
include deletion, addition, or alteration of a limited
number of amino acids. In particular, for use as a
probe, only a substantial portion, and not all of the
15   DNA sequence, is required to be effective. Similarly,
for use in a vaccinia-borne vaccine, only an
"immunologically effective" portion--i.e., sufficient to
generate a protein capable of raising neutralizing
antibodies--is required. So long as the altered amino
20   acid sequence functions immunologically in the same
fashion as the corresponding sequence depicted, it is
included in the designation.

It should be understood that "derived from",
when referring to a DNA sequence or amino acid sequence,
25   indicates a correspondence in composition to the
referenced material, and not necessarily actual physical
derivation therefrom. For example, a DNA encoding a
signal sequence which is derived from influenza
hemagglutinin refers to a DNA constructed so as to
30   encode a peptide sequence substantially similar to that
which is found in influenza HA, and that DNA sequence
may be constructed by, for example, obtaining cDNA from
the mRNA encoding the protein, by synthetic methods
using automated oligonucleotide synthesis, or in any

other manner designed to recreate a coding sequence for
the desired peptide.

B.   General Method

Based on the genomic information provided by
the invention, three general approaches to preparing
vaccines are used: in one, partial or full-length HA
and NA cDNA clones are recombined into a nonessential
region of vaccinia virus, which then express the
inserted EIV antigen coding sequences. These vaccinia
serve as complete EIV vaccines. In a second approach,
synthetic peptides designed from deduced amino acid
sequences of equine HA, if necessary made immunogenic by
size enhancement, are used to prepare vaccines. These
vaccines are used to boost the protective immune
response from animals previously given a single dose of
killed whole virus vaccine. Alternatively, the
synthetic peptide vaccines may be given as a primer
prior to the inoculation with whole virus. In a third
approach, full-length, properly processed HA or NA
proteins are used as vaccines.

HA and NA are used as the basis for active
components of the claimed vaccines because in the case
of humans, it is believed that immunity is conferred by
response to these corresponding surface proteins (Couch,
R.B., et al. Ann Rev Microbiol (1983) 527-549). In
addition, in horses, antisera raised against whole EIV
show high titers of antibody specific against HA of the
same serotype strain.

B.1.   Cloning and Sequencing of the EIV Genes

It is known that the genomes of influenza virus
consist of eight segments of single-stranded RNA, each
of which encodes a particular viral protein. The eight

segments of RNA from each EIV strain were separated by
electrophoresis, and the RNA which encodes HA and that
which encodes NA were isolated. cDNA libraries prepared
from total viral RNA by conventional methods were

5   screened using reverse transcripts of these RNA isolates
and influenza-specific synthetic oligonucleotides as
probes. The cDNA clones thus obtained are useful in
future preparation of corresponding cDNA for use in
vaccines from EIV isolates which have been altered by

10   genetic drift. They thus provide initial probes which
enable the production of a series of vaccines
corresponding to the inevitable evolutionary drift of
the virus.

         The positively hybridizing cDNA clones were

15   then sequenced and their identity as HA and NA genes was
confirmed by the similarity of the deduced amino acid
sequence to known HA and NA proteins. The deduced amino
acid sequences were analyzed for antigen sites using
secondary structure computer profiles, and by localizing

20   sites known to be important in the disease process,
e.g., receptor binding sites.

         The resulting cDNAs encoding HA and NA are
useful as diagnostic probes for the detection of
influenza in horses, as well as in formulating

25   vaccinia-borne vaccines. The probes are used to detect
the EIV genome in biological samples such as blood or
urine by their ability to hybridize to the target
polynucleotide under stringent conditions.

30   B.2. Generation of Recombinant EIV-Vaccinia
               Viruses

         The parameters for effecting the integration of
foreign DNA into vaccinia are known in the art and are
currently in a position for practical utilization.

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Briefly, the desired immunogen is transferred into a
nonessential portion of the vaccinia genome by
coinfecting host cells with both native vaccinia and a
carrier plasmid which contains the foreign gene

5   sandwiched between sequences homologous with the
selected nonessential portion of the vaccinia genome.
In addition, the foreign gene is provided with a
vaccinia promoter, which will permit its expression
under the influence of the vaccinia transcription and

10   translation systems. A general purpose vector capable
of housing the foreign gene has been disclosed by Moss,
B., et al (Proc Natl Acad Sci (USA) (1983) 80:7155-
7159). A diagram of this vector, pGS20, and its manner
of use to obtain a recombinant vaccinia virus containing

15   a desired foreign gene is shown in Figure 5.

pGS20 has a vector fragment derived from
pBR328, a vector compatible with E. coli which contains
the E. coli origin of replication and the ampicillin
resistance gene. The vector contains the promoter from

20   the 7.5 k gene of vaccinia (a gene encoding a 7500
dalton protein), excised as a 275 bp HincII/RsaI
fragment (Venkatsen, et al, Cell (1981) 25:805-813),
which is translocated into the EcoRI site of the
vaccinia thymidine kinase (TK) gene. Other restriction

25   site modifications have been made for convenience, and
there are BamHI and SmaI restriction sites immediately
downstream of the promoter to permit foreign gene
cloning (Mackett, M., et al, supra, incorporated herein
by reference). Figure 6 shows the nucleotide sequence

30   in the region of the promoter/restriction site fragment
junction.

In preparing the vectors of the invention, the
DNA encoding the desired immunogen is inserted into
pGS20 using the restriction sites downstream of the

promoter.   In the present invention this DNA is derived
from equine H7, H3, N7, or N8 and comprises an
immunologically effective portion thereof.   The
recombination vector is amplified in E. coli using

5   transformation to Amp^R and then coinfected along with
wild-type vaccinia into CV-1 cells.   Unlike other large
DNA animal viruses, vaccinia transcribes and replicates
its genome in the cytoplasm of infected cells.   Many of
the enzymes involved in its nucleic acid metabolism,

10   such as DNA and RNA polymerases, enzymes to cap,
methylate, and polyadenylate RNA, as well as thymidine
kinase, are encoded in its own genome.   Indeed,
protein-free vaccinia is noninfective--since it encodes
its own transcriptase and apparently cannot use the

15   transcriptase used by its eukaryotic host, it cannot
synthesize required proteins using its DNA alone in
combination with the host cell machinery.   Cells having
been transformed with both the recombinant, for example
pGS20-derived, vector and vaccinia virus mediate

20   recombination of the DNA portion contained within the
vaccinia-corresponding segments of the vector into the
vaccinia genome.   Use of the TK-encoding sequences to
effect recombination is particularly desirable, as not
only is this a nonessential portion of the vaccinia

25   genome, but a selectable marker is provided for cells
containing the recombined vaccinia--i.e., when the
foreign DNA is inserted, the TK gene is inactivated, and
the tk- recombinant viruses can be selected by plaque
assay on tk- cells in the presence of

30   5-bromodeoxyuridine (BUdR).   As shown in Figure 5, tk-
recombinant virus plaques can be selected yielding the
desired recombinant vaccinia.   Additionally, tk-
vaccinia have been shown to be 10^5 to 10^6 times less
virulent in animals as compared to wild-type vaccinia.
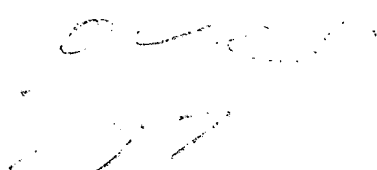
indicating the potential for an increased safety factor
in humans (Smith, G.L., and Moss, B., Biotechniques
(1984) 306-312).

5   While pGS20 is a convenient illustrative
vector, it is understood that alternative constructions
involving other vaccinia promoters in other nonessential
regions of the gene may be used (Moss, et al, Gene
Amplification Analysis, Vol. III, Pappas, T.K., et al,
eds. (1982) New York, Elsevier, pp. 201-213; Mackett,
10   M., et al, supra). Not only CV-1, but any competent
cells susceptible to vaccinia infection can be used to
effect the recombination.

B.3.   Preparation of Synthetic Peptides

15   Peptides were identified as representing
antigenic regions of the HA viral proteins by analogy to
the corresponding regions in the human influenza
proteins, by analysis of the predicted secondary
structure of the equine proteins, and by location of key
20   functional areas in the equine proteins. These
potential antigenic determinants were synthesized using
the solid-state Merrifield method. As these peptides
are often too small to be immunogenic, their size is
enhanced either by polymerization or by conjugation to a
25   carrier protein.

A variety of techniques is available in the art
for such polymerization or conjugation. The most
commonly used polymerization technique employs
glutaraldehyde and effects self-condensation of the
30   peptide. However, conjugation to a carrier protein is,
generally, a preferred approach. Such conjugation is
effected by linking agents which attach to each member
of the conjugate. For example, there are a large number
of hetero bifunctional agents which generate a disulfide

link with one component and a peptide link with the
other. These have been extensively used. Any of the
synthetic peptides disclosed herein may be prepared with
an additional cysteine to permit this mode of linkage.
5   The most popular such linking agent is
N-succinidyl-3-(2-pyridylthio) propionate (SPDP). Other
such agents, which create a disulfide linkage at one end
and an amide linkage through an ε amino on a lysine at
the other end, are available. See, for example, Immun
10   Rev (1982) 82:185. Still other bifunctional coupling
agents form a thioether rather than a disulfide
linkage. Many of these thioether-forming agents are
commercially available, and include reactive esters of
6-maleimidocaproic acid, 2-bromoacetic acid,
15   2-iodoacetic acid, and the like. Carboxyl groups of
these acids can be activated by combining them with
succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium
salt. In addition, reagents which operate through
dehydration, such as dicyclohexylcarbodiimide, can be
20   used.

B.4.   Preparation of Recombinant HA or NA
        Vaccines
              In addition to the vaccines prepared by
25   obtaining recombinant vaccinia virus and by size
enhancement of synthetic peptides, larger proteins
derived from the HA or NA proteins of the equine
influenza viral coat proteins can be prepared. As the
entire genome encoding these proteins is available,
30   suitable DNA encoding these proteins in their entirety
or such smaller portions thereof which are
immunologically effective may be excised from cDNA and
ligated into expression vectors for production of these
proteins in foreign hosts. While it is conceivable that

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bacterial hosts can be used, the production of these
proteins in mammalian cells lines, or even yeast cells,
is preferred, since post-translational processing
available in these hosts more accurately simulates that
5   ordinarily carried out in cells infected with the
virus. Therefore the recombinant proteins produced
would be expected to more closely mimic the viral
proteins as ordinarily found by a target cell.

Suitable host mammalian and yeast cells, and
10 appropriate control sequences and transfer vectors
effective in such hosts, are found in C.5 below. In
general, the desired portion of the protein-encoding DNA
sequence is excised from the gene using suitable
restriction enzymes, and modified, if necessary, prior
15 to ligating into transfer vectors providing operable
control sequences. The ligated vectors are then
transformed into the corresponding hosts, and the hosts
cultured under conditions known to foster the
multiplication of the host cell, and, usually, followed
20 by inducing conditions to activate the control sequences
provided. The recombinant HA and NA proteins are then
retrieved from the cells, by lysis, if necessary, and
purified using standard techniques to obtain-the pure
recombinant protein.

25   In addition, the desired recombinant protein
may be produced in cell culture using co-infection with
vaccinia virus along with the appropriate recombinant
pGS20 derivative (Cochran, et al, _Proc Natl Acad Sci_
(USA) (1985) _82_:19-23).

30

B.5. _Vaccine Preparation_

The vaccines of the invention which are
recombined vaccinia virus are employed in the
conventional manner. Vaccinia virus can be cultured in

standard media, as described below, or by infecting live
animals and harvesting the virus from the lesions. The
administration of the vaccinia is generally by means of
scratching the skin, and non-sterile conditions are
5   often acceptable.

For vaccines which contain peptides or proteins
as active ingredients, administration is slightly more
complex. Typically, such vaccines are prepared as
injectables, either as liquid solutions or suspensions;
10   solid forms suitable for solution in, or suspension, in,
liquid prior to injection may also be prepared. The
preparation may also be emulsified. The active
immunogenic ingredient is often mixed with excipients
which are pharmaceutically acceptable and compatible
15   with the active ingredient. Suitable excipients are,
for example, water, saline, dextrose, glycerol, ethanol,
or the like, and combinations thereof. In addition, if
desired, the vaccine may contain minor amounts of
auxiliary substances such as wetting or emulsifying
20   agents, pH buffering agents, or adjuvants which enhance
the effectiveness of the vaccine. The vaccines are
conventionally administered parenterally, by injection,
for example, either subcutaneously or intramuscularly.
Additional formulations which are suitable for other
25   modes of administration include suppositories,
intranasal aerosols, and, in some cases, oral
formulations. For suppositories, traditional binders
and carriers may include, for example, polyalkylene
glycols or triglycerides; such suppositories may be
30   formed from mixtures containing the active ingredient in
the range of 0.5% to 10%, preferably 1%-2%. Oral
formulations include such normally employed excipients
as, for example, pharmaceutical grades of manitol,
lactose, starch, magnesium stearate, sodium saccharin,

cellulose, magnesium carbonate, and the like. These
compositions take the form of solutions, suspensions,
tablets, pills, capsules, sustained release
formulations, or powders, and contain 10%-95% of active
ingredient, preferably 25%-70%.

The peptides' proteins may be formulated into
the vaccine as neutral or salt forms. Pharmaceutically
acceptable salts include the acid addition salts (formed
with the free amino groups) and which are formed with
inorganic acids such as, for example, hydrochloric or
phosphoric acids, or such organic acids as acetic,
oxalic, tartaric, mandelic, and the like. Salts formed
with the free carboxyl groups may be derived from from
inorganic bases such as, for example, sodium, potassium,
ammonium, calcium, or ferric hydroxides, and such
organic bases as isopropylamine, trimethylamine,
2-ethylamino ethanol, histidine, procaine, and the like.

The peptide or protein-based vaccines are
administered in a manner compatible with the dosage
formulation, and in such amount as will be
therapeutically effective and immunogenic. The quantity
to be administered depends on the subject horse to be
treated, capacity of the subject's immune system to
synthesize antibodies, and the degree of protection
desired. Precise amounts of active ingredient required
to be administered depend on the judgment of the
practitioner and are peculiar to each subject. However,
suitable dosage ranges are of the order of several
hundred micrograms active ingredient per subject.
Suitable regimes for initial administration and booster
shots are also variable, but are typified by an initial
administration followed in one or two week intervals by
a subsequent injection or other administration.
Alternatively, primer doses of the vaccines of the

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invention may be used one to two weeks before injection
with conventional killed virus vaccine.

Administration is typically by injection
intramuscularly, but the peptides or proteins may also
5 be formulated for intranasal application.

A preferred use of the synthetic peptide
immunogens is that they be incorporated into existing
immunization regimens as either a first or "priming"
inoculation or as second and subsequent "boosting"
10 inoculations in combination with other vaccines. It has
been shown by others that a single dose of a synthetic
peptide "primes" the immune system such that subsequent
inoculation with the whole virus (e.g., poliovirus)
elicits extremely high titers of neutralizing
15 antibodies. The practice of priming or boosting with
synthetic peptides offers two major advantages: it
lessens the number of whole virus vaccine inoculations
(and the accompanying side effects) required to achieve
immunity, and it retains at least one dose of whole
20 virus vaccine in the immunization regimen, thus allowing
the animal's immune system an opportunity to see the
entire antigenic repertoire of the pathogen.

C. Standard Methods
25   C.1. Preparation of cDNA
First-strand cDNA was synthesized using
RNA-dependent DNA polymerase from avian myeloblastosis
virus (AMV), according to Ulrich, A., et al, Science
(1977) 196:1313-1319, using an appropriate primer. RNA
30 template was removed by denaturation at 100°C for five
minutes followed by chilling on ice and centrifugation
at 5000 rpm for 10 min at room temperature. The
supernatant was treated with DNA polymerase I, large
fragment to synthesize the second strand. The hairpin

loop was cleaved with S1 nuclease from <u>Aspergillus</u>
<u>oryzae</u> in 300 mM NaCl, 30 mM NaOAc, pH 4.5, 3 mM $ZnCl_2$
for 30 min at 37°C with 600 units of enzyme. After
extraction of cDNA with phenol/chloroform, small

5   oligonucleotides were removed by three ethanol
precipitations in the presence of ammonium acetate,
i.e., using 1/2 volume 7.5 M ammonium acetate and two
volumes of ethanol at -70°C. The resulting blunt-ended,
double-stranded cDNA can then be fractionated either

10 using gel filtration through a Sepharose 4B (Pharmacia)
or by ultracentrifugation in 5-20% gradients of glycerol
in 100 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 1 M EDTA,
followed by fractionation of the gradient.

The double-stranded DNA was then cloned into

15 suitable vectors using homopolymeric tailing essentially
as described by Sutcliffe, J.G., <u>Nucleic Acid Res</u> (1978)
<u>5</u>:2721-2732.

Thus, to complete cDNA libraries in pBR322,
double-stranded cDNAs were tailed with dCTP using a

20 reaction containing 0.2 M potassium cacodylate, 25 mM
Tris, pH 6.9, 2 mM dithiothreitol, 0.5 mM $CoCl_2$, 200
mM dCTP, 400 µ/ml BSA, and 40 units calf thymus
terminal transferase for 5 min at 22°C. After
extraction with phenol/chloroform and ethanol

25 precipitation the dC-tailed cDNA was annealed with
PstI-digested pBR322 tailed with oligo-dG using 2.5 µg
pBR322 per 1 µg cDNA/ml. The annealed mixture was
transferred into <u>E. coli</u> 1061 using the $CaCl_2$
treatment of Casabadan, N., et al, <u>J Mol Biol</u> (1980)

30 <u>138</u>:179-207.

C.2.   <u>Screening cDNA Libraries</u>
cDNA libraries were screened by replicating
plates onto duplicate nitrocellulose filters (S&S BA-85)

and permitting the colonies to grow at 37°C for 14-16 hr
on L-agar containing 15 μg/ml tetracycline, followed
by 12-24 hr on L-agar containing 170 μg/ml
chloramphenicol. Colonies were lysed with 10% SDS, and
5   the DNA fixed to the filter by sequential treatment for
5 min with 0.5 M NaOH, 1.5 M NaCl, then 0.5 Tris-HCl, pH
8.0, 1.5 M NaCl, followed by 2x standard saline citrate
(1x SSC = 0.5 M NaCl, 0.105 M sodium citrate). Filters
were air-dried and baked at 80°C for 2 hr in vacuo.
10   For nick-translated probe, the duplicate
filters are prehybridized at 42°C for 16-18 hr with 10
ml per filter of DNA hybridization buffer (50% formamide
(40% formamide if reduced stringency), 5x SSC, pH 7.0,
5x Denhardt's solution (polyvinylpyrrolidine, plus
15   Ficoll and bovine serum albumin; 1x = 0.02% of each), 50
mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 50
μg/ml yeast tRNA, and 50 μg/ml denatured and sheared
salmon sperm DNA).
           Samples are hybridized with nick-translated DNA
20   probes at 42°C for 12-36 hr for homologous species and
37°C for heterologous species contained in 5 ml of this
same DNA hybridization buffer. The filters are washed
two times for 30 min, each time at 50°C, in 0.2x SSC,
0.1% SDS for homologous species hybridization, and at
25   50°C in 3x SSC, 0.1% SDS for heterologous species
hybridization. Filters are air dried and
autoradiographed for 1-3 days at -70°C.
           For synthetic (12-30 mer) oligonucleotide
probes, the duplicate filters are prehybridized at 42°C
30   for 2-8 hr with 10 ml per filter of oligo-hybridization
buffer (6x SSC, 0.1% SDS, 1 mM EDTA, 5x Denhardt's,
0.05% sodium pyrophosphate and 50 μg/ml denatured and
sheared salmon sperm DNA).
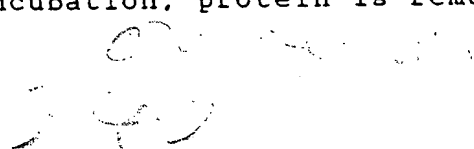
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The samples are hybridized with kinased
oligonucleotide probes of 12-30 nucleotides under
conditions which depend on the composition of the
oligonucleotide. Typical conditions employ a
5   temperature of 30-42°C for 24-36 hr with 5 ml/filter of
this same oligo-hybridization buffer containing probe.
The filters are washed two times for 15 min at 23°C,
each time with 6x SSC, 0.1% SDS and 50 mM sodium
phosphate buffer at pH 7, then are washed once for 2 min
10   at the calculated hybridization temperature with 6x SSC
and 0.1% SDS, air dried, and are autoradiographed at
-70°C for 2 to 3 days.

         C.3.   Vector Construction and Verification
15   Construction of suitable vectors containing the
desired coding and control sequences employs standard
ligation and restriction techniques which are well
understood in the art. Isolated plasmids, DNA
sequences, or synthesized oligonucleotides are cleaved,
20   tailored, and religated in the form desired.
         Site specific DNA cleavage is performed by
treating with the suitable restriction enzyme (or
enzymes) under conditions which are generally understood
in the art, and the particulars of which are specified
25   by the manufacturer of these commercially available
restriction enzymes. See, e.g., New England Biolabs,
Product Catalog. In general, about 1 μg of plasmid or
DNA sequence is cleaved by one unit of enzyme in about
20 μl of buffer solution; in the examples herein,
30   typically, an excess of restriction enzyme is used to
insure complete digestion of the DNA substrate.
Incubation times of about one hour to two hours at about
37°C are workable, although variations can be
tolerated. After each incubation, protein is removed by

extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be

5   performed by polyacylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

        Restriction cleaved fragments may be blunt

10   ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 5-10 µM

15   dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the

20   nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or Bal-31 results in hydrolysis of any single-stranded portion.

25   Synthetic oligonucleotides are prepared by the method of Efimov, V.A., et al (Nucleic Acids Res (1982) 6875-6894), and can be prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for

30   labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7

pmoles γ32P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Ligations are performed in 15-50 µl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM MgCl$_2$, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 µM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na$^+$ and Mg^{+2} using about 1 unit of BAP or CIP per µg of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Ligations for plasmid construction are confirmed by first transforming E. coli strain MC1061 obtained from Dr. M. Casadaban (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are

selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared
5   according to the method of Clewell, D. B., et al, _Proc Natl Acad Sci (USA)_ (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D. B., _J Bacteriol_ (1972) 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of
10   Sanger, F., et al, _Proc Natl Acad Sci (USA)_ (1977) 74:5463 as further described by Messing, et al, _Nucleic Acids Res_ (1981) 9:309, or by the method of Maxam, et al, _Methods in Enzymology_ (1980) 65:499.

15   C.4   Site-Specific Mutagenesis

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide
20   complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the
25   resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.
30   Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer and then washed at a temperature which permits

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hybrids of an exact match to remain, but at which the
mismatches with the original strand are washed off.
Plaques which remain hybridized to the probe at the
stringent wash temperature are then picked, cultured,
5   and the DNA recovered.

For probing, plaques are screened by
replicating the plaques onto duplicate nitrocellulose
filter papers (S & S type BA-85) and infected cells are
allowed to grow at 37°C for 14-16 hr on L agar
10   containing 15 µg/ml tetracycline. The colonies are
lysed with 10% SDS and the DNA is fixed to the filter by
sequential treatment for 5 min with 500 mM NaOH/1.5 M
NaCl, then 0.5 M Tris HCl(pH 8.0)/1.5 M NaCl followed by
2x standard saline citrate (SSC). Filters are air dried
15   and baked at 80°C for 2 hr.

For synthetic (12-30 mer) oligonucleotide
probes, the duplicate filters are prehybridized at 42°C
for 2-8 hr with 10 ml per filter of oligo-hybridization
buffer (6x SSC, 0.1% SDS, 1 mM EDTA, 5x Denhardt's,
20   0.05% sodium pyrophosphate and 50 µg/ml denatured and
sheared salmon sperm DNA).

The samples are hybridized with kinased
oligonucleotide probes of 12-30 nucleotides under
conditions which depend on the composition of the
25   oligonucleotide. Typical conditions employ a
temperature of 30-42°C for 24-36 hr with 5 ml/filter of
this same oligo-hybridization buffer containing probe.
The filters are washed two times for 15 min at 23°C,
each time with 6x SSC, 0.1% SDS and 50 mM sodium
30   phosphate buffer at pH 7, then are washed once for 2 min
at the stringent wash temperature with 6x SSC and 0.1%
SDS. Typically, the stringent wash temperature for
oligonuceotides of 16-24 bases with from 1 to 3
mismatches will be 40-70°C, and can most easily be

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determined by successive washes of the hybridized
filter. For example, the hybridized filters can be
washed first at 40°C, then at 50°C, then at 60°C, and
then at 70°C, with air drying of the filter and
5   autoradiography at -70°C overnight between each wash.

A shorter modification of this procedure was
particularly useful in the construction of mutated
sequences containing new restriction sites. In this
modification, the sequence to be modified is excised and
10   inserted into M13 vectors as a single strand and the
preparation is treated with Klenow in the presence of
primer to create a heteroduplex, as in the above
conventional procedure. The insert is then removed from
the phage vector and cloned into a convenient host
15   plasmid, such as a pUC or pBR plasmid for
amplification. After amplification the plasmid DNA is
cut with a restriction enzyme protocol which includes
the enzyme recognizing the desired new site. Only the
mutagenized double-stranded plasmids yield the correct
20   length fragment. These fragments are isolated, for
example on gels, and no probing is necessary.

C.5.   Hosts and Control Sequences

Both procaryotic and eucaryotic systems may be
25   used to express the EIV encoding sequences; procaryotic
hosts are the most convenient for cloning procedures.
Procaryotes most frequently are represented by various
strains of E. coli; however, other microbial strains may
also be used. Plasmid vectors which contain replication
30   sites and control sequences derived from a species
compatible with the host are used; for example, E. coli
is typically transformed using derivatives of the pUC
series or pBR322. pBR322, for example, contains genes
for ampicillin and tetracycline resistance, and thus

provides additional markers which can be either retained
or destroyed in constructing the desired vector.
Commonly used procaryotic control sequences which are
defined herein to include promoters for transcription
5   initiation, optionally with an operator, along with
ribosome binding site sequences, include such commonly
used promoters as the beta-lactamase (penicillinase) and
lactose (lac) promoter systems (Chang, et al, _Nature_
(1977) _198_:1056 and the tryptophan (trp) promoter system
10   (Goeddel, et al _Nucleic Acids Res_ (1980) _8_:4057 and
hybrid control systems such as those employing upstream
trp and downstream lac regions.
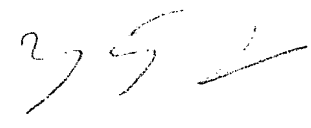
Eucaryotic hosts are often preferred because of
their ability to effect post-translational processing.
15   Microbial eucaryotic hosts include yeast; laboratory
strains of _Saccharomyces cerevisiae_, Baker's yeast, are
most used although a number of other strains are
commonly available. Vectors employing, for example, the
2 μ origin of replication of Broach, J. R., _Meth Enz_
20   (1983) _101_:307, or other yeast compatible origins of
replications (see, for example, Stinchcomb, et al,
Nature (1979) _282_:39, Tschempe, et al, _Gene_ (1980)
_10_:157 and Clarke, L, et al, _Meth Enz_ (1983) _101_:300)
may be used. Control sequences for yeast vectors
25   include promoters for the synthesis of glycolytic
enzymes (Hess, et al, _J Adv Enzyme Req_ (1968) _7_:149;
Holland, et al, _Biochemistry_ (1978) _17_:4900).
Additional promoters known in the art include the
promoter for 3-phosphoglycerate kinase (Hitzeman, et al,
30   _J Biol Chem_ (1980) _255_:2073), and those for other
glycolytic enzymes.

Eucaryotic host cell cultures derived from
multicellular organisms may be used also. These systems
have the additional advantage of the ability to splice

out introns and thus can be used directly to express
genomic fragments. Useful host cell lines include VERO
and HeLa cells, and Chinese hamster ovary (CHO) cells.
Expression vectors for such cells ordinarily include
5   promoters and control sequences compatible with
mammalian cells such as, for example, the commonly used
early and late promoters from Simian Virus 40 (SV 40)
(Fiers, et al, *Nature* (1978) *273*:113), or other viral
promoters such as those derived from polyoma, Adenovirus
10   2, bovine papiloma virus, or avian sarcoma viruses. The
controllable promoter, hMTII (Karin, M., et al, *Nature*
(1982) *299*:797-802) may also be used. General aspects
of mammalian cell host system transformations have been
described by Axel: U.S. Patent No. 4,399,216 issued 16
15   August 1983. It now appears, also that "enhancer"
regions are important in optimizing expression; these
are, generally, sequences found upstream or downstream
of the promoter region in non-coding DNA regions.
Origins of replication may be obtained, if needed, from
20   viral sources. However, integration into the chromosome
is a common mechanism for DNA replication in
eucaryotes.
        Depending on the host cell used, transformation
is done using standard techniques appropriate to such
25   cells. The calcium treatment employing calcium
chloride, as described by Cohen, S. N., *Proc Natl Acad
Sci (USA)* (1972) *69*:2110, or the RbCl$_2$ method
described in Maniatis, et al, *Molecular Cloning: A
Laboratory Manual* (1982) Cold Spring Harbor Press, p.
30   254 may be used for procaryotes or other cells which
contain substantial cell wall barriers. For mammalian
cells without such cell walls, the calcium phosphate
precipitation method of Graham and van der Eb, *Virology*
(1978) *52*:546, optionally as modified by Wigler, M., et

al. Cell (1979) 16:777-785 may be used. Transformations
into yeast may be carried out according to the method of
Van Solingen, P., et al. J Bact (1977) 130:946 or of
Hsiao, C. L., et al. Proc Natl Acad Sci (USA) (1979)
5   76:3829.

        C.6.   ELISA Assay for Antibodies Against Test
                Peptides

        The synthetic peptides of the invention and
10   their size-enhanced derivatives were used to immunize
rabbits and mice, and their ability to raise antibodies
in sera was evaluated using an enzyme-linked
immunosorbent assay (ELISA). The appropriate peptide
for reaction with the antisera was bound to 96-well
15   plates by pretreating the wells in 2% glutaraldehyde for
2 hr at room temperature and then incubating a solution
of the appropriate peptide (100 μl of 50 μg
peptide/ml PBS) in the wells for 2 hr at 37°C. After
washing, the wells received fourfold dilutions of
20   antisera ranging from 1/20 to 1/327,680, and the plates
were incubated for 2 hr at 37°C. After washing, the
wells received a 1/3500 dilution of horseradish
peroxidase-conjugated goat anti-rabbit immunoglobulin
(Boehringer-Mannheim) in 0.1% ovalbumin and 0.05%
25   Tween-20 in PBS. The plates were incubated at 4°C
overnight. After washing, a solution of 0.1% citric
acid, pH 5.0, 0.04% o-phenyldiamine, and 0.012% peroxide
was added to each well, and the plates were incubated
for 5-30 min at room temperature. The color reaction
30   was stopped by adding 4 N HCl and the absorbance at 490
nm was read on a Biotek ELISA Reader, Model EL-308.
Titers for each serum were determined by plotting
absorbance against serum dilution for both preimmune and
immune samples.

C.7 Plaque Reduction Assay

The ability of antisera to neutralize virus was
tested using a plaque reduction assay. Preimmune sera
were used as controls. Antisera to be tested and
5 control antisera were heat-treated at 56°C for 30 min in
order to inactivate nonspecific antiviral agents present
in all mammalian sera. 100-150 plaque-forming units
(pfu) of EIV were incubated with an equal volume of
appropriately diluted control and test sera (usually
10 twofold dilutions) for 1 hr at 25°C. Monolayers of MCDK
cells were then inoculated with the virus/serum mixture
and adsorption allowed to take place for 1 hr at room
temperature. The monolayers were then rinsed with
Earl's basic salt solution and overlaid with a mixture
15 of DMEM and 0.8% agarose containing 2.5 µg/ml
trypsin. The wells were incubated in a 5% CO_2
atmosphere at 37°C for 4 days in order allow plaque
formation by viable virus. Cells were stained with
crystal violet and total plaques recorded. The serum
20 neutralization titer was determined as the dilution of
the serum that reduced EIV pfu by greater than 50%.

D.   Examples

The following examples are intended to
25 illustrate the invention, but not to limit its scope.

D.1.   Preparation and Sequencing of Equine HA
        and NA cDNA

Both known EIV strains were used as sources of
30 RNA encoding the desired proteins. Any fresh isolate
from a diseased animal can be used as starting material:
both EIV-A1 and EIV-A2 are pandemic in the diseased
equine population. In the examples below, EIV-A1 stain
(H7N7) was originally isolated from an infected horse in

Florida in 1973 and passed in four isolation ponies at
Cornell University before being propagated in
Madin-Darby canine kidney (MDCK) cells. It is
designated A1/Cornell/16/74. The EIV-A2 strain (H3N8),

5   designated A2/Kentucky/1/81, was obtained as an
allantoic fluid preparation. Both stocks were shown to
produce clinically recognizable disease in horses.

These strains, propagated in MDCK cells, were
concentrated by centrifugation and purified by banding

10   twice in 30%/60% discontinuous sucrose gradients. The
purified virus was treated with 10μg/ml DNase at 23°C
for twenty minutes, and the virions were disrupted using
0.5% SDS followed by two extractions with
phenol:chloroform:isoamyl alcohol (50:50:1) and two

15   extractions with chloroform:isoamyl alcohol (50:1). The
aqueous phase was adjusted to 0.3 M sodium acetate, and
RNA was precipitated with 2-1/2 volumes absolute ethanol.

Preparation of cDNA Probes

20   To isolate RNA fragments for cDNA probe
preparation, 1.5-2 μg viral RNA was denatured in 10 mM
methylmercuric hydroxide for 1.5 minutes at 65°C and
then electrophoresed on a 1.4% agarose gel in
Tris-borate buffer (0.089 M Tris, 0.089 M boric acid,

25   0.02 M EDTA) at 12 V/cm for two hours. The gel was
stained using 0.5 μg/ml ethidium bromide. The RNA
bands identified as HA or NA (by size analogy to human
influenza models) were then used to prepare cDNA probes
by the method of Lonberg and Gilbert, Proc Natl Acad Sci

30   (USA) (1983) 80:3661-3665. Briefly, 5-10 μl agarose
plugs were removed from the appropriate areas of the gel
with 50 μl capillary tubes and the plugs melted at
65°C for three minutes and cooled to 37°C. First strand
cDNA was obtained using RNA-dependent DNA polymerase, as

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in section C.1.   Calf thymus DNA was used as a random
primer.

Preparation of cDNA Libraries Containing HA and NA

5

cDNA libraries in pBR322 were prepared from
total RNA of each strain.   The cDNA libraries were
prepared as described above using two primer methods.
In the first method, oligo-dT 12-18mers were used as

10   primers after addition of poly-A tails to the viral
negative stranded poly-A deficient RNA.   Three μg of
viral RNA was incubated with E. coli poly-A polymerase
in a reaction containing 50 mM Tris-HCl, pH 7.6, 10 mM
$MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 10 mM ATP for 3

15   min at 37°C and then extracted with phenol/chloroform
and ethanol-precipitated.   The resulting extended RNA
was used as template.

In the alternative method, the synthetic
oligomer 5'-AGCAAAAGCAGG-3' (oligo a), which is a known

20   complement to twelve nucleotides in the 3' terminal
untranslated region of each of the eight individual
viral RNAs in all influenza viruses was used as primer.
A second synthetic primer 5'-AGTAGAAACAAGG-3' (oligo b),
which complements an influenza consensus sequence in the

25   5' untranslated region at the upstream end of each
coding region in the viral RNA, was used to prime the
complementary strand.   Three μg viral RNA yielded
approximately 1 μg cDNA size selected to 500 bp; and
each library contained about 500,000 independent

30   recombinants.

For probing with cDNA probes, hybridization
buffer contained 50% formamide, 5x SSC, 50 mM HEPES, pH
8.0, 5x Denhardt solution (1x Denhardt's = 0.02% each
polyvinyl pyrollidine, Ficoll, and BSA), 0.1% SDS, 50

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mM/ml yeast tRNA, and 50 mM sheared and denatured salmon
sperm DNA. For probing with synthetic probes, the
hybridization buffer contained 6x SSC, 0.1% SDS, 1 mM
EDTA, 5x Denhardt's, 0.05% sodium pyrophosphate, and 15
5   µg/ml denatured and sheared salmon sperm DNA.

Isolation of HA and NA cDNA Sequences

The cDNA libraries were probed using one of the
reverse-transcribed RNA for each of the desired H7, H3,
10   N7, and N8 sequences prepared as described above and
using the two synthetic oligomers used for priming in
the reverse-transcribed synthesis, as also described
(oligos a and b). For reverse-transcribed RNA,
prehybridization was at 42°C for 12-14 hr, and
15   hybridization was at 42°C for 12-36 hr using 10^5
cpm/filter. For probing with oligos a or b,
prehybridization was at 42°C for 2-8 hours and
hybridization, at 33°C for 6-12 hours using 10^6
cpm/filter·kinased probe. (The 33°C temperature was
20   calculated using the formula $T_H = 4[G + C] + 2 [A + T]$
-3°C. Suggs, S.V., et al, Developmental Biology Using
Purified Genes (1981), ed. D.D. Brown and C.F. Fox,
Academic Press, New York, pp. 683-693.) For the RNA
probe, the filters were washed twice for 30 min each at
25   50°C in 0.2x SSC, 0.1% SDS, and then air dried and
autoradiographed for 1-3 days at -70°C. For the oligo
probes the filters were washed twice for 2 min at 23°C
each, with 6x SSC, 0.1% SDS, and 0.05% sodium
pyrophosphate, then for 2 min at 33°C in the same
30   buffer, and then air dried and autoradiographed at -70°C
for 1-2 days. A large number of positive clones were
obtained from both EIV-A1 and EIV-A2 libraries, but only
one clone from each library which hybridized with all

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three probes in each of the four cases was retained for
further study.

The resulting clones representing H7, H3, N7,
and N8 were sequenced by subcloning into M13 mp8, mp9,
5   mp18, and mp19 (Messing, J., et al, _Gene_ (1982)
_19_:269-276) following sequence determination using the
dideoxy method of Sanger, et al, _Proc Natl Acad Sci_
(USA) (1977), _74_:5463-5467. The nucleotide sequence of
the cDNA sequences for H7, H3, N7, and N8 are shown in
10   Figures 1-4.

Examination of the sequences in Figures 1-4
indicates the characteristic features of HA1 and HA2
proteins from H7 and H3, including conserved cysteines,
clip sites between HA1 and HA2, and the conserved
15   membrane fusion region at the amino terminus of HA2.

D.2.   Synthesis of Synthetic Peptides Based HA
        Sequence Information

The following peptides were synthesized using
20   the method of Merrifield, J., _J Amer Chem Soc_ (1963)
_85_:2149-2154.

Based on the EIV-A1 (H7):

(A1-X) representing residues 146-173: Ala-Cys-
Arg-Arg-Ser-Arg-Ser-Ser-Phe-Tyr-Ala-Glu-Met-Lys-Trp-Leu-Leu-
Ser-Asn-Thr-Asp-Asn-Gly-Val-Phe-Pro-Gln-Met.
25

(A1-Y) representing residues 181-209: (Cys)-Lys-
Arg-Glu-Pro-Ala-Leu-Ile-Ile-Trp-Gly-Ile-His-His-Ser-Gly-
Ser-Thr-Ala-Glu-Gln-Thr-Arg-Leu-Tyr-Gly-Ser-Gly-Asn-Lys-(Cys)

(A1-Z) representing residues 309-337: Arg-Ala-
30   Ile-Gly-Lys-Cys-Pro-Arg-Tyr-Val-Lys-Gln-Lys-Ser-Leu-Met-
Leu-Ala-Thr-Gly-Met-Lys-Asn-Val-Pro-Glu-Asn-Ser-Thr.

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For EIV-A2:

(A2-X) representing residues 143-159: Thr-Gly-Val-Thr- Gln-Asn-Gly-Arg-Ser-Gly-Ala-Cys-Arg-Arg-Gly-Ser-Ala-(Ser)-(Arg).

(A2-Y) representing residues 191-218: Lys-Leu-Tyr-Ile-Trp-Gly-Ile-His-His-Pro-Ser-Thr-Asn-Asn-Glu-Gln-Thr-Lys-Leu-Tyr-Ile-Gln-Glu-Ser-Gly-Arg-Val-Thr.

(A2-Z) representing residues 322-343: Lys-Tyr-Ile-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr-Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Ile.

Finally, a synthetic peptide was designed after the amino terminus of the HA2 portion of both the H7 and H3 hemagglutinins (residues 345-357 in H3 and 350-362 in H7):

(A1/A2-N) Gly-Ile-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-(Lys)-(Lys).

Residues in parenthesis are not present in natural sequence but were added for purposes of peptide conjugation or ease in synthesis.

The foregoing peptides are designated by the shorthand symbols shown, and were selected for synthesis on the following basis: both X-peptides (A1-X and A2-X) and Y-peptides (A1-Y and A2-Y) correspond to regions in the human analogs which are major antigenic sites as mapped by monoclonal antibodies and by sequencing of wild-type variants, and thus represent epitopes which, in the human model, elicit B-cell response.

The Y-peptides and Z-peptides (A1-Z and A2-Z) are derived from areas which, in human analogs, have been shown to be recognized by T-cells, and may thus function in those analogs to elicit a T-helper cell response and/or a cytolytic T-cell response (Lamb, J.R., et al, Nature (1982) 300:66-69; Wabuke-Bunoti, M.A.N., et al, J Immunol (1984) 133:2194-2201). Both Z-peptides

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and the N-peptide are derived from portions which correspond, in the human hemagglutinin, to areas of antigenic stability.

The foregoing peptides were used to immunize rabbits or mice both as prepared and as modified to enhance presentation size in the injected subject. For size enhancement, peptides were either self-polymerized with glutaraldehyde or covalently linked to keyhole limpet hemocyanin (KLH) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

For polymerization with glutaraldehyde, 4-10 mg/ml peptide was incubated at 21°C for 2 hr in 50 mM NaHPO$_4$ (pH 7.5), 150 mM glutaraldehyde. Insoluble products resulted after one or more hours, and the precipitate was resuspended and mixed with Freund's adjuvant for immunization.

For conjugation to KLH, peptide at 4-10 mg/ml was incubated at 4°C for 20 min in the presence of 1 mg/ml carbodiimide in distilled water, adjusted to pH 3.5 with 1 N HCl. KLH was added at 1 mg/ml and the pH of the solution was adjusted to 9.0 with 5 volumes of distilled water containing 1 mM NaOH. The mixture was incubated for 2 hr at 21°C and dialyzed against phosphate-buffered saline at 4°C for 16 hr. After dialysis, the conjugated peptides were mixed with Freund's adjuvant for immunization.

D.3.   Immunization with Synthetic Peptides
Antigenicity

The synthetic peptides were shown to be antigenic in rabbits and mice by ELISA assay of sera obtained from animals injected with each synthetic peptide. The same synthetic peptide was used as the antigen in the ELISA assay of ¶C.6.

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For rabbits, each animal received a total of
two inoculations. The first inoculation was
administered intranodally with complete Freund's
adjuvant. The following booster at three weeks was
5   injected intramuscularly with incomplete Freund's
adjuvant. The animals received 125 μg of free peptide
or polymerized peptide per dose, or 165 μg of KLH
peptide conjugate (20 peptide:1 KLH). The animals were
bled from the ear vein before the first inoculation to
10   obtain preimmune serum, and ten days after each
subsequent inoculation.

For mice, eight-week-old female Balb/C mice
received two inoculations of the same preparations as
were used for rabbits, except that the peptide content
15   in each dose was 20 μg. The first inoculation was
given intramuscularly with complete Freund's adjuvant;
the second, three weeks later, in incomplete adjuvant.
Animals were bled from the eye seven or eight days after
each inoculation.
20   Table 1 shows the results for the Al-X and Al-Y
peptides and their derivatives.

Table 1

	Peptide	Method of Peptide Presentation	Anti-Peptide Antibody Titer
Rabbit 2A1	A1-X	Free	*
Rabbit 2A2	"	Free	1/20,480
Rabbit 2B1	"	Polymerized	1/80
Rabbit 2B2	"	Polymerized	1/80
Rabbit 2C1	"	Conjugated to KLH	1/5120
Rabbit 2C2	"	Conjugated to KLH	1/5120

* Animal died.

Mouse 2fA	A1-X	Free	1/1280
Mouse 2fB	"	Free	1/1280
Mouse 2pA	"	Polymerized	1/320
Mouse 2pB	"	Polymerized	<1/20
Mouse 2cA	"	Conjugated to KLH	1/81,920
Mouse 2cB	"	Conjugated to KLH	1/81,920

Mouse 3fA	A1-Y	Free	1/320
Mouse 3fB	"	Free	<1/20
Mouse 3pA	"	Polymerized	1/80
Mouse 3pB	"	Polymerized	1/80
Mouse 3cA	"	Conjugated to KLH	1/320
Mouse 3cB	"	Conjugated to KLH	1/320

These data indicate that the synthetic peptides of this invention will elicit moderate to high antibody titers when administered in free and/or size-enhanced form.

Use of the Synthetic Peptides as Immunogenic Boosters

Eight-week-old Balb/C mice received single intramuscular injections of 100 µl of Equicine (Bayvet, Miles Laboratories). Equicine is a killed virus vaccine containing both A1 and A2 EIV serotypes. Three weeks later, the mice received either a second dose of Equicine or a mixture containing 15 µg each of the 7 peptides prepared in ¶D.2 in incomplete Freund's adjuvant. Six days after the second inoculation, the animals were bled from the eye, and the sera were

assayed for neutralizing activity against EIV-Al virus
in a plaque reduction assay.
The results are given in Table 4.

5

Table 4

Animal	Boost	Plaque reduction titer (EIV-Al)
10A	Equicine	1/160
10B	Equicine	1/80-1/160
11A	EIV peps	1/40
11B	"	>1/320
11C	"	>>1/320
11D	"	1/20
11E	"	>1/320
11F	"	1/80

10

15   The results show that the EIV peptides were as, or more,
effective in 4 of 6 animals than additional killed virus
in maintaining the level of neutralizing
plaque-reduction antibodies. The same results may be
achieved when the titer against EIV-A2 virus is analyzed.

20   A second similar experiment was performed to
determine whether EIV synthetic peptides could be used
in an adjuvant other than incomplete Freund's to boost
neutralizing titer after a single inoculation of
Equicine. The adjuvant preparation selected is a form

25   of Halvogen™, an adjuvant used in Equicine vaccine and
approved for equine usage. It has the composition 7%
Tween 20, 3% Arlacel 20, 50% soybean oil, and 1%
Carbopol in H_2O.

            Five groups of three mice each were inoculated

30   with a 100 µl dose of Equicine. Group I received two
boosts of Equicine at three week intervals; Group II
received two boosts of 15 µg each peptide (105 µg
total) in incomplete Freund's adjuvant (IFA); Group III
received two boosts of 15 µg each peptide in the above

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Halvogen™ adjuvant; Group IV received two boosts of 15
µg each peptide in aqueous solution (no adjuvant);
Group V received no boosts. The mice were bled eight
days after the second boost; the sera in each group were
5   pooled, heat treated, and assayed in the plaque
reduction assay. The results are presented in Table 5.

Table 5

Group	Boost	Plaque reduction titer (EIV-A1)
I	Equicine	1/640
II	EIV peps in IFA	1/1280
III	EIV peps in Halvogen	1/640
IV	EIV peps in aqueous (no adjuvant)	1/320
V	None	1/160

The results show that EIV peps in IFA boosted
plaque reduction titer after a single primary vaccine
inoculation better than subsequent vaccine boosts. In
addition, EIV peps in Halvogen boosted as well as
20   vaccine boosts although EIV peps without adjuvant did
not. Pooled sera from groups I, II, and III all showed
at least a fourfold rise in titer over pooled sera from
animals receiving no boost (group V). A fourfold titer
increase is generally regarded as "seroconversion" in
25   the evaluation of vaccine preparations. Similar results
may be obtained when the plaque reduction titer against
EIV-A2 is analyzed.

In a converse experiment, 8-week-old female
Balb/C mice received a single intramuscular injection of
30   a mixture containing 15 µg of each of the above
peptides in complete Freund's adjuvant, while controls
received similar inoculations of a heterologous peptide
derived from unrelated viral sources (bovine viral
diarrhea virus, or BVDV). Three weeks later, the mice

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received 10^5 pfu of UV-irradiated EIV-A1 or EIV-A2
intramuscularly in incomplete Freund's adjuvant. The
animals were bled six days after the second inoculation
and the sera analyzed as above. The results are shown
in Table 6.

Table 6

Animal no. and initial treatment		Plaque reduction titer (EIV-A1)
7D	(EIV peps)	1/40
7A	(EIV peps)	>1/10*
8B	(BVD peps)	<1/10
8A	(BVD peps)	<1/10
9B	-0-	<1/10

*Assay not carried beyond 1/10 dilution.

These results show that the EIV peptides can
also specifically prime the immune system of an animal
for a subsequent inoculation of whole virus whereas an
injection of heterologous peptide does not. Similar
results may be obtained against EIV-A2.

D.4.   Preparation of Recombinant Vaccinia
        Viruses Bearing Equine Influenza Antigens

Methods described by Mackett, et al (supra).
were used to insert H7, H3, N7, and N8 into vaccinia
carrier.

Each cDNA was first subcloned into pGS20 which
contains the 7.5 k vaccinia gene promoter with unique
BamHI and SmaI sites immediately downstream for the
insertion of the desired heterologous gene, and flanked
by vaccinia thymidine kinase (TK) gene. The tk- gene
permits homologous recombination into the vaccinia
genome and affords a method of selection (generation of
tk-viruses).

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The constructions of the intermediate pGS20
derivative vectors containing EIV inserts are shown in
Figure 6 and are described below. The nucleotide
numbering referred to in the text corresponds to that in
5   Figures 1-4. In order to place the initiation codons of
each EIV cDNA as close to the vaccinia promoter as
possible and to prevent possible interference with
processing, the cDNAs were prepared for insertion by
removal of homopolymeric dC or dG tracts that were
10 generated during cDNA cloning and by providing
convenient restriction sites proximal to the 5' and 3'
ends.

        For H7, the H7 cDNA was cloned into the PstI
site of M13mp7, thus generating a SalI site at the 3'
15 end of the DNA. A BamHI site was placed 3 nucleotides
before the ATG start codon by converting the nucleotide
at position 40 from A to C using site-specific
mutagenesis. The H7 coding sequence was removed as a
BamHI/SalI (blunted) fragment and cloned into the
20 BamHI/SmaI digested pGS20.

        For H3, nucleotides 1-94 and 1720-1797 were
removed from H3 cDNA by digestion with AccI at the 5'
end (GTCTAC) and BglI at the 3' end (GCCAAAAAGGC). The
deleted sequences were replaced using synthetic
25 oligomers which contained SmaI restriction sites 8
nucleotides 5' of the ATG and 3 nucleotides 3' to the H3
termination codon while retaining the same coding
sequence as represented in Figure 2. The resulting cDNA
was removed as a SmaI/SmaI fragment and cloned into the
30 SmaI site of pGS20.

        For N7, the full-length cDNA was cloned into
pUC9 so that a unique SmaI site was positioned at the 3'
end. The upstream portion of the sequence was excised
by treating with HindIII and EcoRI and inserted into

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HindIII/EcoRI digested M13mp9. This upstream sequence
was modified to create an NdeI site by conversion of
nucleotides 56 from G to C and 58 from A to T using
site-specific mutagenesis. The heteroduplex phage was
5   digested with HindIII and EcoRI and the heteroduplex
fragment was amplified in pBR322, then removed by
treating with NdeI, blunting with Klenow, and digesting
with EcoRI. The mutated excised fragment was ligated
into SmaI/EcoRI digested pUC19. The SmaI site from
10   pUC19 is destroyed by the ligation and a unique BamHI
site 5' to the N7 ATG results. The modified N7 5'
fragment was removed from pUC19 as a BamHI/EcoRI
fragment and religated to the remainder of the N7
sequence contained in pUC9 at the unique EcoRI site.
15   The desired sequence containing the entire N7 coding
region was obtained as a BamHI/SmaI fragment and ligated
into BamI/SmaI-digested pGS20.

For N8, the cDNA was cloned into the PstI site
of pUC8, which placed a unique SmaI site 3' of the
20   cDNA. A unique AhaIII site already exists in N8 3
nucleotides 5' of ATG, and hence an AhaIII/SmaI digest
generates a full-length cDNA which is blunt-end ligated
into the SmaI site of pGS20.

The four vaccinia-EIV vectors described above
25   are designated pGS20-H7, pGS20-H3, pGS20-N7, and
pGS20-N8, or, collectively, pGS20-EIV. Figure 6 shows
the nucleotide sequence surrounding the coding region of
each EIV cDNA inserted into the BamHI/SmaI cloning site
of pGS20. The dotted line after each ATG represents
30   the sequence described in Figures 1-4 to the terminal
PstI cloning sites (after the poly-G tracts) with the
exception of H3, which was modified as described in the
text.
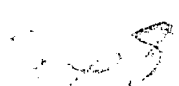
These recombinant vectors are transfected into
cells infected with wild-type vaccinia virus, which was
purchased from Wyeth Laboratories, Inc. (Marietta, PA)
and plaque purified twice in CV-1 cells. A small
5   aliquot of virus stock (0.1 ml) is diluted with an equal
volume of trypsin (0.25 mg/ml) and incubated for 30 min
at 37°C with vortexing, followed by sonication to
disperse any cell clumps. The virus is diluted to a
concentration of 5x 10^4 plaque-forming units per ml in
10   phosphate-buffered saline (PBS) containing 0.2% bovine
serum albumin (BSA) and penicillin/steptomycin. CV-1
cells are infected in monolayers on 60 mm plates with 1
ml vaccinia virus to give a multiplicity of infection of
0.05 pfu/cell. The virus inoculum is incubated on the
15   cells for 2 hr at 37°C with rocking.

The DNA for transformation is prepared as
described in Graham, et al. _Virology_ (1973) _52_:456;
Stow, et al, _J Gen Virol_ (1976) _33_:447; and Frost, et
al, _Virology_ (1978) _91_:39. Briefly, 5-10 µg of
20   plasmid (pGS20 derivative) DNA, and 1-2 µg wild-type
vaccinia virus DNA are added to 1 ml to Hepes-buffered
saline (0.14 molar NaCl, 5 mM KCl, 1 mM Na phosphate,
0.1% dextrose, 20 mM Hepes, pH 7.05), and 50 µl of 2.5
molar $CaCl_2$ is added. The solutions are mixed and
25   left at room temperature for 30 min, and the desired
precipitate of DNA forms in this time.

The virus inoculum is aspirated from the CV-1
cell layers, and 1 ml of the DNA precipitate is
substituted and the layers left at room temperature for
30   30 min, after which 9 ml prewarmed Eagle's MEM
containing 8% FBS is added. The layers are incubated
for 3.5 hr at 37°C, before aspirating off the medium and
replacing it with 10 ml fresh Eagle's MEM containing 8%
FBS. The monolayers are then left at 37°C for 2 days,

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resulting in the development of vaccinia cytopathic
effects. The cells and virus are harvested by scraping,
spun down, and resuspended in 0.5 ml Eagle's MEM and
frozen at -20°C.

5   The resuspended virus/cell pellets are
freeze-thawed three 'times by freezing the cells at -20°C
for 30 minutes and quickly thawing them at 37°C,
followed by sonication for one minute to disperse the
virus/cell clumps. The resulting crude virus stock is
10   then inoculated in serial 10-fold dilutions onto 143
cells (Mackett, M., et al, $\underline{J Virol}$ (1984) $\underline{49}$:857-864), a
human tk- cell line. After two hours at 37°C, the 143
monolayers are then overlaid with 1% agarose containing
1x modified Eagle's medium, 5% fetal bovine serum, and
15   25 μg/ml 5-bromodeoxyuridine (BUdR). After incubation
for two days at 37°C, BUdR-resistant plaques are picked
and grown for 48 hours at 37°C in 24 well plates of 143
cell monolayers in the presence of 25 μg/ml BUdR.

          Virus is harvested and assayed for the presence
20   of the EIV-derived gene by DNA-DNA dot blot
hybridization, as follows: cells are scraped from the
dish into an Eppendorf centrifuge tube, centrifuged for
1 minute, and the cell pellet resuspended in 0.2 ml
PBS. After freeze-thawing 3 times and sonicating as
25   described above, the sonicate is applied to a
nitrocellulose filter and air dried. A wild-type virus
control is also spotted onto the same filter. The
filter is then placed on paper soaked in (1) 0.5 M NaOH,
(2) 1 M Tris-HCl, pH 7.5, and (3) 2x SSC, for 5 minutes
30   each. The filter is then baked at 80°C under vacuum for
2 hours. The baked filter is prehybridized for 1-4
hours at 42°C in 5 ml 50% formamide, 4x SSC, 5x
Denhardt's solution, and 0.1 mg/ml sheared and boiled
salmon sperm DNA. The filter is then hybridized

overnight at 42°C to 2x 10^7 cpm of ^{32}P-labeled EIV
gene probe in 5 ml prehybridization buffer. The probe
is prepared by isolating 5 μg of EIV gene insert from
one of the pGS20-derivative vectors and nick translating
5   the DNA using a commercially available nick translation
kit. The filter is then washed twice for 30 minutes in
0.5x SSC, 0.1% SDS, air dried, and autoradiographed
overnight at -70°C.

Virus containing EIV gene inserts by dot blot
10   hybridization are infected onto monolayers of 143 cells
and left until a confluent cytopathic effect is
obtained. The culture medium is then aspirated off and
the cells lysed in 1% SDS, 0.1 M ß-mercaptoethanol, 50
mM Tris-HCl, pH 7.8. The lysate is made 0.5 mg/ml in
15   proteinase K, incubated 4 hours at 37°C, phenol
extracted, ethanol precipitated, and analyzed by
restriction enzyme analysis to show that the vaccinia
genomes indeed contain the desired EIV genes.

The recombinant vaccinia so generated were
20   designated vac-H7, vac-H3, vac-N7 and vac-N8 or
collectively, vac-EIV.

D.5   Expression of EIV Surface Antigens by Vaccinia
      Recombinants

25   Each vac-EIV recombinant was plaqued separately
onto CV1 monolayers. The monolayers were washed, fixed,
and incubated with rabbit anti-EIV-A1 (for vac-H7 and
vac-N7) and rabbit anti-EIV-A2 (for vac-H3 and vac-N8).
Antibody binding specificially to EIV surface antigens
30   was detected by a subsequent incubation with
^{125}I-labeled staphylococcal A protein followed by
autoradiography. In each case, all recombinant virus
plaques bound the appropriate antibody.

Immunoprecipitation of ^{35}S-labeled cellular
lysates obtained from monolayers of CV1 cells infected
by individual vac-EIV recombinants showed the presence
of EIV protein. In the case of each recombinant virus,
5   antisera raised against the appropriate whole virus
immunoprecipitated a characteristic hemagglutinin or
neuraminidase molecule identified by polyacrylamide gel
electrophoresis and autoradiography.

10   D.6   Amplification of Vaccinia Recombinants
       After the vac-EIV recombinants were identified
as both carrying the appropriate EIV gene and expressing
the encoded EIV protein, a single plaque of each is
isolated x2 in 143 cells in the presence of BUdR. The
15   twice purified isolate is amplified once in 143 cells in
the presence of BUdR and subsequently in CV-1 cells in
the absence of selective pressure. The resulting
vac-EIV recombinants are stable genetically and can be
further amplified in CV-1 cells.

20

D.7   Bioassay of Vaccinia Recombinants
       Horses are used as subjects to assess the
ability of the recombinant vaccinia to raise titers of
neutralizing anti-EIV antibodies in serum.
25   In general, pairs of horses are inoculated with
wild-type or recombinant virus by intradermal
administration of 1-2x 10^8 plaque-forming units
distributed in 2-3 sites on the back, and are bled at
days 0, 14, and 28. The sera are then tested for the
30   presence of anti-EIV neutralizing antibodies in the
plaque reduction assay of ¶C.7.
       In one experiment, four horses, 1-3 years old,
housed in a P3 facility at Washington State University,
Pullman, Washington, were ascertained to be serum

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negative with respect to anti-EIV antibodies, but were
otherwise undocumented with respect to exposure.
Control and test virus were administered intradermally
by scarification with a needle to bleeding at four

5   locations on the animals' necks. Each animal received a
total of 10^8 pfu of each indicated virus in a total of
0.4 ml (0.1 ml at each scar). VSV was used as a
control; test viruses were H7, H3, N7, and N8, as
described above (¶D.4).

10   The animals were bled at day 0 (preimmune), and
at days 7, 14, and 21, and their sera assessed for
neutralizing antibodies against each of EIV-A1 and
EIV-A2 using the plaque reduction assay described in
¶C.7 hereinabove. The results, shown below in Table 7,

15   indicate that while control VSV was not able to raise
antiserum against EIV, all combinations of recombinant
vaccinia containing DNA encoding H and/or N peptides,
.were able to do so.

20   Table 7

Horse	Vaccine	EIV Strain	Day 0	7	14	21
Lola	VSV	A1	1/10	1/10	1/10	1/10
		A2	<1/10	<1/10	<1/10	<1/10
Howard	N7,N8	A1	1/10	1/20	1/160	1/160
		A2	<1/10	1/40	1/160	1/160
Fanny	H7,H3	A1	<1/10	1/160	1/320	1/160
	N7,N8	A2	<1/10	1/320	1/640	1/320
Leo	H7,H3	A1	<1/10	1/800	1/1600	1/800
		A2	<1/10	1/640	1/1280	1/640

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The results are maximum serum dilutions able to reduce EIV pfu by 50%. The results for Leo at days 7, 14, and 21 are based on an initial serum dilution of 1/50 rather than the 1/10 dilution used for all other

5   results.

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Claims

1.   A recombinant DNA sequence encoding a
protein selected from the group consisting of equine
5 hemagglutinin H7, equine hemagglutinin H3, equine
neuraminidase N7, and equine neuraminidase N8.

2.   A vaccine effective against influenza in
equine hosts which comprises at least one DNA sequence
10 of claim 1 or an immunologically effective portion
thereof recombinantly integrated into vaccinia virus.

3.   A method of immunizing horses against
influenza which comprises administering the vaccine of
15 claim 2.

4.   A peptide useful in preparing a vaccine
against equine influenza which comprises an amino acid
sequence selected from the group consisting of:
20

Ala-Cys-Arg-Arg-Ser-Arg-Ser-Ser-Phe-Tyr-Ala-
Glu-Met-Lys-Trp-Leu-Leu-Ser-Asn-Thr-Asp-Asn-Gly-Val-Phe-
Pro-Gln-Met;

Cys-Lys-Arg-Glu-Pro-Ala-Leu-Ile-Ile-Trp-Gly-Ile-H
25 is-His-Ser-Gly-Ser-Thr-Ala-Glu-Gln-Thr-Arg-Leu-Tyr-Gly-
Ser-Gly-Asn-Lys-Cys;

Arg-Ala-Ile-Gly-Lys-Cys-Pro-Arg-Tyr-Val-Lys-
Gln-Lys-Ser-Leu-Met-Leu-Ala-Thr-Gly-Met-Lys-Asn-Val-Pro-
Glu-Asn-Ser-Thr;

30 Thr-Gly-Val-Thr-Gln-Asn-Gly-Arg-Ser-Gly-Ala-
Cys-Arg-Arg-Gly-Ser-Ala-Ser-Arg;

Lys-Leu-Tyr-Ile-Trp-Gly-Ile-His-His-Pro-Ser-
Thr-Asn-Asn-Glu-Gln-Thr-Lys-Leu-Tyr-Ile-Gln-Glu-Ser-
Gly-Arg-Val-Thr;

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Lys-Tyr-Ile-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr-
Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Ile; and
                Gly-Ile-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-
Gly-Lys-Lys.

5

        5.   A vaccine for immunizing horses against
influenza wherein the peptide of claim 4 is optionally
linked to a neutral carrier or polymerized.

10   6.   A method of immunizing horses against
influenza wherein the protocol is selected from
                boosting subject horses previously
administered another vaccine against EIV with a vaccine
comprising at least one peptide of claim 4; and
15   administering a primer dose of a vaccine
comprising at least one peptide of claim 4 to horses
which are subsequently administered another vaccine
against EIV..

20   7.   Recombinantly produced equine hemagglutinin
H3, equine hemagglutinin H7, equine neuraminidase N7, or
equine neuraminidase N8.

                8.   A vaccine effective for immunizing horses
25 against influenza which comprises a protein selected
from the group consisting of equine hemagglutinin H3,
equine hemagglutinin H7, equine neuraminidase N7, and
equine neuraminidase N8 or an immunologically effective
portion thereof.

30
                9.   A cDNA sequence substantially equivalent to
a sequence selected from the group consisting of the
equine H7 of Figure 1, the equine H3 of Figure 2, the
equine N7 of Figure 3, and the equine N8 of Figure 4.

        10. A method for preparing a vaccine against
equine influenza which comprises probing a cDNA library
prepared from a fresh isolate of EIV from a desired
animal with a probe derived from the cDNA sequence of
5   claim 9.

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EIV - A1 hemagglutinin (H7)

```
                                              ctgcaggggg ggggggggg gggagcaaaa gcaggggata cata
                                                      ┌─► HA1
        50                                            ┌─100
ATG AAC ACT CAA ATT CTA ATA TTA GCC ATT TCG GCA TTC CTC TGT GTA CGT GCA GAT AAA ATC TGC CTA GGA CAT CAT GCT GTG TCT AAT
MET Asn Thr Gln Ile Leu Ile Leu Ala Ile Ser Ala Phe Leu Cys Val Arg Ala Asp Lys Ile Cys Leu Gly His His Ala Val Ser Asn
                                                                             ▲                                        30

        150                                           200
GGA ACC AAA GTA GAC ACC CTT ACT GAA AAG GGA ATA GAA GTT GTC AAT GCA ACA GAA ACA GTT GAA CAA AAA AAC ATC CCC AAG ATC TGC
Gly Thr Lys Val Asp Thr Leu Thr Glu Lys Gly Ile Glu Val Val Asn Ala Thr Glu Thr Val Glu Gln Lys Asn Ile Pro Lys Ile Cys
                                                                                                                      60▲

        250                                           300
TCA AAA GGG AAA CAG ACT ATT GAC CTT GGT CAA TGT GGA TTA CTA GGG ACC ACT ATT GGT CCC CCC CAA TGC GAC CAA TTT CTT GAA TTC
Ser Lys Gly Lys Gln Thr Ile Asp Leu Gly Gln Cys Gly Leu Leu Gly Thr Thr Ile Gly Pro Pro Gln Cys Asp Gln Phe Leu Glu Phe
                                ▲                                                        ▲                            90

        350                                           400
TCT GCT AAT TTA ATA ATT GAG AGA AGA GAA GGT GAT GAC ATT TGT TAT CCA GGC AAA TTT GAC AAT GAA GAA ACA TTG AGA CAA ATA CTC
Ser Ala Asn Leu Ile Ile Glu Arg Arg Glu Gly Asp Asp Ile Cys Tyr Pro Gly Lys Phe Asp Asn Glu Glu Thr Leu Arg Gln Ile Leu
                                                    ▲                                                                 120

        450
AGA AAA TCC GGA GGA ATT AAA AAG GAG AAT ATG GGA TTC ACA TAT ACC GGA GTG AGA ACC GAT GGA GAG ACT AGC GCC TGT AGA AGG TCA
Arg Lys Ser Gly Gly Ile Lys Lys Glu Asn MET Gly Phe Thr Tyr Thr Gly Val Arg Thr Asp Gly Glu Thr Ser Ala Cys Arg Arg Ser
                                                                                                    ▲                150

        500                                           550
AGA TCT TCC TTT TAT GCA GAA ATG AAA TGG CTC CTA TCC AAC ACA GAC AAT GGG GTA TTC CCA CAA ATG ACA AAA TCC TAC AAG AAC ACT
Arg Ser Ser Phe Tyr Ala Glu MET Lys Trp Leu Leu Ser Asn Thr Asp Asn Gly Val Phe Pro Gln MET Thr Lys Ser Tyr Lys Asn Thr
                                                                                                                     180

        600                                           650
AAG AGG GAG CCA GCT CTG ATA ATC TGG GGA ATC CAC CAC TCA GGA TCA ACC GCT GAA CAG ACT AGA TTG TAT GGA AGC GGA AAC AAG TTG
Lys Arg Glu Pro Ala Leu Ile Ile Trp Gly Ile His His Ser Gly Ser Thr Ala Glu Gln Thr Arg Leu Tyr Gly Ser Gly Asn Lys Leu
                                                                                                                     210

        700                                           750
ATA ACA GTT TGG AGT TCC AAA TAC CAA CAA TCT TTT GCC CCA AGC CCT GGA CCA AGG CCG CAA ATA AAT GGC CAA TCA GGA AGA ATT GAC
Ile Thr Val Trp Ser Ser Lys Tyr Gln Gln Ser Phe Ala Pro Ser Pro Gly Pro Arg Pro Gln Ile Asn Gly Gln Ser Gly Arg Ile Asp
                                                                                                                     240

                                    800                                           850
TTT TAC TGG CTG ATG TTA GAT CCC AAT GAT ACT GTT ACT TTC AGT TTT AAT GGG GCC TTT ATA GCA CCT GAC CGC GCC AGT TTT CTA AGA
Phe Tyr Trp Leu MET Leu Asp Pro Asn Asp Thr Val Thr Phe Ser Phe Asn Gly Ala Phe Ile Ala Pro Asp Arg Ala Ser Phe Leu Arg
                                                                                                                     270

                                          900
GGT AAA TCT CTA GGA ATT CAG AGT GAC GCA CAA CTT GAC AAC AAT TGT GAA GGT GAA TGT TAT CAT ATT GGA GGT ACT ATA ATT AGC AAC
Gly Lys Ser Leu Gly Ile Gln Ser Asp Ala Gln Leu Asp Asn Asn Cys Glu Gly Glu Cys Tyr His Ile Gly Gly Thr Ile Ile Ser Asn
                                                              ▲              ▲                                       300

        950                                  1000
TTG CCC TTT CAA AAC ATT AAT AGC AGA GCA ATT GGG AAA TGC CCC AGA TAC GTA AAG CAA AAA AGC TTA ATG CTA GCA ACC GGA ATG AAA
Leu Pro Phe Gln Asn Ile Asn Ser Arg Ala Ile Gly Lys Cys Pro Arg Tyr Val Lys Gln Lys Ser Leu MET Leu Ala Thr Gly MET Lys
                                                      ▲                                                               330

                    1050 ┌─HA1                                 ┌─► HA2 1100
AAT GTT CCT GAA AAT TCT ACA CAC AAA CAG TTA ACT CAT CAC ATG CGC AAA AAA AGA GGT TTA TTT GGT GCA ATA GCA GGA TTT ATT GAA
Asn Val Pro Glu Asn Ser Thr His Lys Gln Leu Thr His His MET Arg Lys Lys Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu
                            ↑                                               ↑└─ conserved HA2 NH2 terminus ─────── 360┘
                    clip?  1150                                            clip

AAT GGA TGG GAA GGA TTA ATA GAT GGA TGG TAT GGA TAC AGA CAT CAG AAT GCA CAA GGA GAA GGA ACT GCT GCA GAC TAC AAA AGT ACA
Asn Gly Trp Glu Gly Leu Ile Asp Gly Trp Tyr Gly Tyr Arg His Gln Asn Ala Gln Gly Glu Gly Thr Ala Ala Asp Tyr Lys Ser Thr
                                                                                                                     390

                                  1250                                           1300
CAA TCT GCT ATC AAT CAA ATA ACC GGG AAA TTA AAC AGA CTA ATA GAA AAA ACC AAC CAG CAA TTT GAA CTA ATA GAT AAT AAA TTC AAT
Gln Ser Ala Ile Asn Gln Ile Thr Gly Lys Leu Asn Arg Leu Ile Glu Lys Thr Asn Gln Gln Phe Glu Leu Ile Asp Asn Lys Phe Asn
                                                                                                                     420

                                          1350
GAA ATA GAA AAG CAA ATT GGC AAT GTT ATT AAC TGG ACT AGA GAT TCT ATC ATC GAA GTA TGG TCA TAT AAT GCA GAA TTC CTC GTG GCA
Glu Ile Glu Lys Gln Ile Gly Asn Val Ile Asn Trp Thr Arg Asp Ser Ile Ile Glu Val Trp Ser Tyr Asn Ala Glu Phe Leu Val Ala
                                                                                                                     450

        1400                                  1450
GTG GAG AAT CAA CAC ACT ATT GAT TCA ACT GAT TCA GAG ATG AAC AAA TTA TAT GAA AAG GTA AGA AGA CAG CTG ACA GAA AAT GCT CAC
Val Glu Asn Gln His Thr Ile Asp Ser Thr Asp Ser Glu MET Asn Lys Leu Tyr Glu Lys Val Arg Arg Gln Leu Arg Glu Asn Ala Glu
                                                                                                                     480

        1500                                  1550
GAA GAT GGT AAT GGC TGT TTT GAA ATA TTC CAC CAA TGT GAC AAT CAT TGC ATC GCC AGC ATT AGA AAC AAT ACA TAT GAT CAT AAA AAA
Glu Asp Gly Asn Gly Cys Phe Glu Ile Phe His Gln Cys Asp Asn His Cys Ile Ala Ser Ile Arg Asn Asn Thr Tyr Asp His Lys Lys
                ▲                                   ▲               ▲                                                510

        1600                                  1650
TAC ACA AAG GAC GCA ATA CAA AAC AGA ATT CAG ATT GAT GCA GTA AAG TTC AGC AGC GGT TAC AAA GAT ATA ATA CTT TGG TTT AGC TTC
Tyr Arg Lys Glu Ala Ile Gln Asn Arg Ile Gln Ile Asp Ala Val Lys Leu Ser Ser Gly Tyr Lys Asp Ile Ile Leu Trp Phe Ser Phe
                                                                                                                     540

                            1700                                    1750 ┌─HA2─┐
GGG GCA TCA TGT TTC TTA TTT CTT GCC ATT GCA ATG GTT CTT GCT TTC ATA TGC ATA AAA AAT GGA AAC ATC CGG TGC ACT ATT TGT ATA TAA
Gly Ala Ser Cys Phe Leu Phe Leu Ala Ile Ala MET Val Leu Ala Phe Ile Cys Ile Lys Asn Gly Asn MET Arg Cys Thr Ile Cys Ile
        ▲                                               ▲                   ▲               ▲ 570

                            1808
gct tgaaaaaaca cccttgtttt ctactccccc ccccccccc ccccttgcag
```

FIG. 1

EIV - A2 hemagglutinin (83)

ctgcagggggg gggggggggg gggggagcaaa agcagggggat acttctgtca atc   50

```
                                              Acc I     100   ┌─HA 1
                                               ↓
ATG AAG ACA ACC ATT ATT TTG ATA CTA CTG ACC CAT TGG GTC TAC AGT CAA AAC CCA ACC AGT GGC AAC AAC ACA GCC ACA CTA TGT CTG
MET Lys Thr Thr Ile Ile Leu Ile Leu Leu Thr His Trp Val Tyr Ser Gln Asn Pro Thr Ser Gly Asn Asn Thr Ala Thr Leu Cys Leu  30
                                                                                                               ▲

          150                                              200
GGA CAC CAT GCA GTA GCA AAT GGA ACA TTG GTA AAA ACA ATA ACT GAT GAC CAA ATT GAG GTG ACA AAT GCT ACT GAA TTA GTT CAG AGC
Gly His His Ala Val Ala Asn Gly Thr Leu Val Lys Thr Ile Thr Asp Asp Gln Ile Glu Val Thr Asn Ala Thr Glu Leu Val Gln Ser  60

              250                                              300
ACT TCA ATA GGG AAA ATA TGC AAC AAC CCA TAT AGG GTT CTA GAT GGA AGA AAC TGC ACA TTA ATA GAT GCA ATG CTA GGA GAT CCC CAC
Thr Ser Ile Gly Lys Ile Cys Asn Asn Pro Tyr Arg Val Leu Asp Gly Arg Asn Cys Thr Leu Ile Asp Ala MET Leu Gly Asp Pro His  90
                     ▲                                              ▲

          350                                              400
TGT GAT GTT TTT CAG TAT GAG AAT TGG GAC CTC TTC ATA GAA AGA AGC AGC GCT TTC AGC AAT TGC TAC CCA TAT GAC ATC CCT GAC TAT
Cys Asp Val Phe Gln Tyr Glu Asn Trp Asp Leu Phe Ile Glu Arg Ser Ser Ala Phe Ser Asn Cys Tyr Pro Tyr Asp Ile Pro Asp Tyr 120
▲                                                         ▲

          450                                              500
GCA TCG CTC CGG TCT ATT GTG GCA TCT TCA GGA ACA TTA GAA TTC ACA GCA GAG GGA TTC ACA TGG ACA GGT GTC ACT CAA AAC GGA AGA
Ala Ser Leu Arg Ser Ile Val Ala Ser Ser Gly Thr Leu Glu Phe Thr Ala Glu Gly Phe Thr Trp Thr Gly Val Thr Gln Asn Gly Arg 150

          550
AGT GGA GCC TGC AGA AGG GGG TCA GCC GAT AGT TTC TTT AGC CGA CTG AAT TGG CTA ACA AAA TCT GGA AAT TCT TAC CCC ACA TTG AAT
Ser Gly Ala Cys Arg Arg Gly Ser Ala Asp Ser Phe Phe Ser Arg Leu Asn Trp Leu Thr Lys Ser Gly Asn Ser Tyr Pro Thr Leu Asn 180
             ▲

     600                                              650
GTA ACA ATG CCT AAC AAT AAC AAT TTC GAT AAA CTA TAC ATC TGG GGG ATC CAT CAC CCG AGC ACA AAC AAT GAG CAG ACA AAA TTG TAT
Val Thr MET Pro Asn Asn Asn Asn Phe Asp Lys Leu Tyr Ile Trp Gly Ile His His Pro Ser Thr Asn Asn Glu Gln Thr Lys Leu Tyr 210

              700                                              750
ATC CAA GAA TCA GGG CGA GTA ACA GTC TCA ACA AAA AGA AGT CAA CAA ACA ATA ATC CCC AAC ATC GGA TCT AGA CCG TGG GTC ACG GGT
Ile Gln Glu Ser Gly Arg Val Thr Val Ser Thr Lys Arg Ser Gln Gln Thr Ile Ile Pro Asn Ile Gly Ser Arg Pro Trp Val Arg Gly 240

          800                                              850
CAA TCA GGC AGG ATA AGC ATA TAT TGG ACC ATT GTG AAA CCT GGA GAT ATC CTA ATG ATA AAC AGT AAT GGC AAC TTA GTT GCA CCG CGG
Gln Ser Gly Arg Ile Ser Ile Tyr Trp Thr Ile Val Lys Pro Gly Asp Ile Leu MET Ile Asn Ser Asn Gly Asn Leu Val Ala Pro Arg 270

              900                                              950
GGA TAT TTT AAA ATG CGA ACA GGG AAA AGC TCT GTA ATG AGA TCA GAT GCA CCC ATA GAC ACT TGT GTG TCC GAC TGT ATT ACA CCA AAT
Gly Tyr Phe Lys MET Arg Thr Gly Lys Ser Ser Val MET Arg Ser Asp Ala Pro Ile Asp Thr Cys Val Ser Glu Cys Ile Thr Pro Asn 300
                                                         ▲              ▲

                                         1000
GGA AGC ATC CCC AAC GAC AAA CCA TTT CAA AAT GTG AAC AAA GTT ACA TAT GGA AAA TGC CCC AAG TAT ATC AAG CAG AAT ACT TTG AAG
Gly Ser Ile Pro Asn Asp Lys Pro Phe Gln Asn Val Asn Lys Val Thr Tyr Gly Lys Cys Pro Lys Tyr Ile Lys Gln Asn Thr Leu Lys 330
                                                                      ▲

                HA1 ◄─┐   ┌─ HA2
          1050                            1100
CTG GCC ACT GGG ATG AGG AAT GTA CCA GAA AAG CAA ATC AGA GGA ATC TTT GGA GCA ATA GCG GGA TTC ATA GAA AAC GGC TGG GAA GGA
Leu Ala Thr Gly MET Arg Asn Val Pro Glu Lys Gln Ile Arg Gly Ile Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly 360
                                                 ↑    └──── conserved HA2 NH2 terminus ────┘
                                               cllp

                    1150                            1200
ATG GTT GAT GGG TGG TAT GGA TTC CGA TAT CAG AAT TCG GAA GGA ACA GGA CAA GCT GCA GAT CTA AAG AGC ACT CAA GCA GCC ATC GAC
MET Val Asp Gly Trp Tyr Gly Phe Arg Tyr Gln Asn Ser Glu Gly Thr Gly Gln Ala Ala Asp Leu Lys Ser Thr Gln Ala Ala Ile Asp 390

          1250                                              1300
CAG ATC AAT GGA AAA TTG AAC AGA GTG ATT GAA AGG ACC AAT GAG AAA TTC CAT CAA ATA GAG AAG GAA TTC TCA GAA GTA GAA GGG ACA
Gln Ile Asn Gly Lys Leu Asn Arg Val Ile Glu Arg Thr Asn Glu Lys Phe His Gln Ile Glu Lys Glu Phe Ser Glu Val Glu Gly Arg 420

              1350                                              1400
ATC CAG GAC TTG GAG AAG TAT GTA GAA GAC ACC AAA ATA GAC CTA TGG TCC TAC AAT GCA CAG TTA CTC GTG GCT CTA GAA AAT CAA CAT
Ile Gln Asp Leu Glu Lys Tyr Val Glu Asp Thr Lys Ile Asp Leu Trp Ser Tyr Asn Ala Gln Leu Leu Val Ala Leu Glu Asn Gln His 450

          1450
ACG ATT GAC TTA ACA GAT GCA GAA ATG AAT AAA TTA TCC GAG AAG ACT AGG CGC CAG TTA AGA GAA AAC GCG GAA GAC ATG GGG GCT GGA
Thr Ile Asp Leu Thr Asp Ala Glu MET Asn Lys Leu Ser Glu Lys Thr Arg Arg Gln Leu Arg Glu Asn Ala Glu Asp MET Gly Gly Gly 480

     1500                                              1550
TGT TTC AAG ATT TAT CAC AAA TGT GAT AAT GCA TGC ATT GGA TCA ATA AGA AAT GGG ACA TAT GAC CAT TAC ATA TAC AGA GAT GAA GCA
Cys Phe Lys Ile Tyr His Lys Cys Asp Asn Ala Cys Ile Gly Ser Ile Arg Asn Gly Thr Tyr Asp His Tyr Ile Tyr Arg Asp Glu Ala 510
 ▲                      ▲                     ▲

              1600                                              1650
TTA AAC AAC CGA TTT CAA ATT AAA GGT GTT GAA TTG AAA TCA GGC TAC AAA GAT TGG ATA CTG TGG ATT TCA TTC GCC ATA TCA TGC TTC
Leu Asn Asn Arg Phe Gln Ile Lys Gly Val Glu Leu Lys Ser Gly Tyr Lys Asp Trp Ile Leu Trp Ile Ser Phe Ala Ile Ser Cys Phe 540
                                                                                                              ▲

             Bgl I                                        HA2 ◄─┐
                 ↓        1700                                 1750
TTA ATT TGC GTT GTT CTA TTG GGT TTC ATC ATG TGG GCT TGC CAA AAA GGC AAC ATC AGA TGC AAC ATT TGC ATT TCA gtaaactga
Leu Ile Cys Val Val Leu Leu Gly Phe Ile MET Trp Ala Cys Gln Lys Gly Asn Ile Arg Cys Asn Ile Cys Ile 564
 ▲                                              ▲                     ▲              ▲

                    1797
tagttaaaaa aaaaaaaaa cccccccccc cctgcag
```

FIG. 2

EIV - A1 neuraminidase (N7)

```
                                                              50
                    ctgcaggggg ggggggggg ggtttttttt tttttagcaa aagcagggta attttgaa

                                                100
ATG AAT CCT AAT CAA AAA CTC TTT GCA TCA TCC GGA ATA GCA ATA GTG CTA GGA ATA ATA AAT CTT CTC ATA GGA ATA TCC AAT ATG AGT
MET Asn Pro Asn Gln Lys Leu Phe Ala Ser Ser Gly Ile Ala Ile Val Leu Gly Ile Ile Asn Leu Leu Ile Gly Ile Ser Asn MET Ser
                                                                                                                      30

150                                             200
TTA AAT ATA TCT CTA TAT TCA AAA GGG GAA AGC CAC AAG AAT AAT AAC CTA ACA TGC ACA AAT ATC AAC CAG AAT GAT ACC ACC ATG GTA
Leu Asn Ile Ser Leu Tyr Ser Lys Gly Glu Ser His Lys Asn Asn Asn Leu Thr Cys Thr Asn Ile Asn Gln Asn Asp Thr Thr MET Val
                                                                                                                      60

            250
AAC ACG TAC ATC AAT AAC GCA ACA ATA ATT GAC AAA AGT ACA AAA ATA GAA AAC CCT GGT TAT CTA CTG CTG AAC AAA AGT CTA TGC AAC
Asn Thr Tyr Ile Asn Asn Ala Thr Ile Ile Asp Lys Ser Thr Lys Ile Glu Asn Pro Gly Tyr Leu Leu Leu Asn Lys Ser Leu Cys Asn
                                                                                                                      90

            350                                             400
GTT GAA GGA TGG GTT GTA ATA GCA AAG GAC AAT GCG ATT AGA TTT GGA GAA AGC GAA CAA ATC ATA GTA ACT AGA GAA CCT TAT GTC TCA
Val Glu Gly Trp Val Val Ile Ala Lys Asp Asn Ala Ile Arg Phe Gly Glu Ser Glu Gln Ile Ile Val Thr Arg Glu Pro Tyr Val Ser
                                                                                                                      120

                            450                                             500
TGT GAT CCT CTA AGT TGC AAA ATG TAT GCT CTA CAC CAA GGT ACT ACA ATC AGA AAC AAG CAT TCA AAT AGT ACC ACA CAC GAC AGA ACA
Cys Asp Pro Leu Ser Cys Lys MET Tyr Ala Leu His Gln Gly Thr Thr Ile Arg Asn Lys His Ser Asn Ser Thr Thr His Asp Arg Thr
                                                                                                                      150

GCC TTC CGA GGG CTC ATT TCT ACT CCA TTA GGT AGC CCC CCA ACA GTG AGC AAC AGT GAA TTC ATA TGT GTT GGG TGG TCA AGC ACA AGC
Ala Phe Arg Gly Leu Ile Ser Thr Pro Leu Gly Ser Pro Pro Thr Val Ser Asn Ser Glu Phe Ile Cys Val Gly Trp Ser Ser Thr Ser
                                                                                                                      180

600                                 650
TGC CAT GAT GGG GTA AAC AGG ATG ACA ATT TGT GTA CAA GGA GAC AAT GAA AAT GCT ACT GCA ACA GTG TAT TAC AAC AAG AGA CTT ACA
Cys His Asp Gly Val Asn Arg MET Thr Ile Cys Val Gln Gly Asp Asn Glu Asn Ala Thr Ala Thr Val Tyr Tyr Asn Lys Arg Leu Thr
                                                                                                                      210

            700                                             750
ACC ACT ATT AAA ACA TGG GCT AAA AAC ATT TTA AGA ACC CAA GAG TCT GAA TGT GTT TGT CAT AAC AGC ACT TGT GTA GTG GTA ATG ACT
Thr Thr Ile Lys Thr Trp Ala Lys Asn Ile Leu Arg Thr Gln Glu Ser Glu Cys Val Cys His Asn Ser Thr Cys Val Val Val MET Thr
                                                                                                                      240

                    800                                             850
GAT GGG CCC GCA AAT AAC CAG GCG TTC ACA AAA GTA ATA TAC TTT CAT AAA GGA ATG ATA ATA AAA GAA GAA TCA CTA AAA GGT TCA GCC
Asp Gly Pro Ala Asn Asn Gln Ala Phe Thr Lys Val Ile Tyr Phe His Lys Gly MET Ile Ile Lys Glu Glu Ser Leu Lys Gly Ser Ala
                                                                                                                      270

            900                                             950
AAA CAC ATA GAA GAA TGT TCT TGT TAT GGT CAT AAT CAA AGA GTG ACT TGT GTC TGC AGA GAC AAC TGG CAG GGT GCA AAT AGA CCT ATT
Lys His Ile Glu Glu Cys Ser Cys Tyr Gly His Asn Gln Arg Val Thr Cys Val Cys Arg Asp Asn Trp Gln Gly Ala Asn Arg Pro Ile
                                                                                                                      300

                            1000
ATA GAG ATT GAC ATG AAT AAA TTG GAA CAT ACA AGT AGA TAT ATA TGC ACA GGG GTA TTA ACA GAC ACC AGT AGA CCC AAG GAT AAA ACA
Ile Glu Ile Asp MET Asn Lys Leu Glu His Thr Ser Arg Tyr Ile Cys Thr Gly Val Leu Thr Asp Thr Ser Arg Pro Lys Asp Lys Thr
                                                                                                                      330

1050                                1100
ATA GGG GAA TGC TTC AAT CCT ATT ACT GGA AGC CCT GGT GCA CCA GGG ATA AAA GGT TTC GGA TTC CTA AAT GAG GAT AAT ACT TGG CTA
Ile Gly Glu Cys Phe Asn Pro Ile Thr Gly Ser Pro Gly Ala Pro Gly Ile Lys Gly Phe Gly Phe Leu Asn Glu Asp Asn Thr Trp Leu
                                                                                                                      360

            1150                                             1200
GGG AGA ACA ATC AGC CCC AGA TTG AGG AGT GGA TTT GAA ATG CTG AAG ATA CCT AAT GCT GGG ACT GAC CCA GAG TCC AAA ATA AAA GAA
Gly Arg Thr Ile Ser Pro Arg Leu Arg Ser Gly Phe Glu MET Leu Lys Ile Pro Asn Ala Gly Thr Asp Pro Glu Ser Lys Ile Lys Glu
                                                                                                                      390

                    1250                                             1300
AGA CAA GAA ATA GTT AGT AAT GAC AAT TGG TCA GGC TAT TCC GGA AGT TTC ATT GAT TAT TGG AAT GAC AAC AGT GAA TGC TAC AAT CCA
Arg Gln Glu Ile Val Ser Asn Asp Asn Trp Ser Gly Tyr Ser Gly Ser Phe Ile Asp Tyr Trp Asn Asp Asn Ser Glu Cys Tyr Asn Pro
                                                                                                                      420

            1350                                             1400
TGT TTC TAT GTA GAA TTA ATT AGA GGA AGG CCT GAA GAA GCA AAA TAT GTT GAA TGG ACC AGT AAC AGC CTA ATT GCA CTA TGT GGG AGC
Cys Phe Tyr Val Glu Leu Ile Arg Gly Arg Pro Glu Glu Ala Lys Tyr Val Glu Trp Thr Ser Asn Ser Leu Ile Ala Leu Cys Gly Ser
                                                                                                                      450

                    1450
CCA ATC TCA GTT GGG TCT GGA TCT TTC CCT GAT GGG GCA CAA ATT AAA TAC TTT TCG TAA aacgaa aaaaacccct gtttctactc ccccccccc
Pro Ile Ser Val Gly Ser Gly Ser Phe Pro Asp Gly Ala Gln Ile Lys Tyr Phe Ser
                                                                        469

    1515
cccccctgcag
```

FIG. 3

XIV - A2 neuraminidase (N8)

ctgcaggggg gggggagcag gagtttaaa

```
                      50                                                        100
ATG AAT CCA AAT CAA AAG ATA ATA GCA ATT GGA TCT GCA TCA TTA GGA ATA CTA ATC CTC AAC GTC ATT CTC CAT GTA GTC AGC ATT ATA
MET Asn Pro Asn Gln Lys Ile Ile Ala Ile Gly Ser Ala Ser Leu Gly Ile Leu Ile Leu Asn Val Ile Leu His Val Val Ser Ile Ile
                                                                                                                       30

                      150                                                       200
GTA ACA GTA CTG GTC CTC AAT AAC AAT GGA ACA GGT CTG AAC TGC AAC GGG ACG ATC ATA AGA GAG TAC AAT GAA ACA GTA AGA GTA GAA
Val Thr Val Leu Val Leu Asn Asn Asn Gly Thr Gly Leu Asn Cys Asn Gly Thr Ile Ile Arg Glu Tyr Asn Glu Thr Val Arg Val Glu
                                                                                                                       60

                      250                                                       300
AGA ATT ACT CAA TGG TAT AAT ACT AAT ACA ATC GAG TAT ATA GAG AGA CCT TCA AAT GAA TAC TAC ATG AAC AAC ACC GAA CCA CTG TGT
Arg Ile Thr Gln Trp Tyr Asn Thr Asn Thr Ile Glu Tyr Ile Glu Arg Pro Ser Asn Glu Tyr Tyr MET Asn Asn Thr Glu Pro Leu Cys
                                                                                                                       90

                      350
GAG GCC CAG GGC TTT GCA CCA TTT TCC AAA GAT AAT GGA ATA CGA ATT GGG TCG AGA GGT CAT GTT TTT GTA ATA AGA GAA CCT TTT GTC
Glu Ala Gln Gly Phe Ala Pro Phe Ser Lys Asp Asn Gly Ile Arg Ile Gly Ser Arg Gly His Val Phe Val Ile Arg Glu Pro Phe Val
                                                                                                                       120

            400                                                       450
TCA TGT TCT CCC TTA GAA TGT AGA ACC TTT TTC CTC ACA CAG GGC TCA TTA CTT AAT GAC AAA CAT TCT AAC GGC ACA GTG AAG GAC CGA
Ser Cys Ser Pro Leu Glu Cys Arg Thr Phe Phe Leu Thr Gln Gly Ser Leu Leu Asn Asp Lys His Ser Asn Gly Thr Val Lys Asp Arg
                                                                                                                       150

                      500                                                       550
AGT CCA TAT AGG ACT TTG ATG AGT GTC AAA GTA GGG CAA TCA CCT AAT GTG TAT CAA GCT AGG TTT GAA TCG GTG GCA TGG TCA GCA ACA
Ser Pro Tyr Arg Thr Leu MET Ser Val Lys Val Gly Gln Ser Pro Asn Val Tyr Gln Ala Arg Phe Glu Ser Val Ala Trp Ser Ala Thr
                                                                                                                       180

                      600                                                       650
GCA TGC CAC GAT GGG AAA AAG TGG ATG ACA GTT GGA GTC ACA GGG CCC GAT AAT CAA GCA GTT GCA GTA GTG AAC TAT GGA GGT GTT CCG
Ala Cys His Asp Gly Lys Lys Trp MET Thr Val Gly Val Thr Gly Pro Asp Asn Gln Ala Val Ala Val Val Asn Tyr Gly Gly Val Pro
                                                                                                                       210

                      700                                                       750
GTT GAT ATC ATT AAT TCA TGG GCA GGG GAT ATC CTA AGA ACC CAA GAA TCG TCA TGC ACC TGC ATT AAA GGA GAC TGT TAT TGG GTG ATG
Val Asp Ile Ile Asn Ser Trp Ala Gly Asp Ile Leu Arg Thr Gln Glu Ser Ser Cys Thr Cys Ile Lys Gly Asp Cys Tyr Trp Val MET
                                                                                                                       240

                      800
ACT GAT GGA CCG GCA AAC AGG CAA GCT AAA TAT AGG ATA TTC AAA GCA AAA GAT GGA AGA ATA ATT GGG CAG ACT GAT ATA AGT TTC AAT
Thr Asp Gly Pro Ala Asn Arg Gln Ala Lys Tyr Arg Ile Phe Lys Ala Lys Asp Gly Arg Ile Ile Gly Gln Thr Asp Ile Ser Phe Asn
                                                                                                                       270

            850                                                       900
GGG GGA CAC ATA GAG GAG TGT TCT TGT TAC CCC AAT GAA GGG AAG GTG GAG TGT GTA TGC AGG GAC AAC TGG ACT GGA ACA AAT AGA CCA
Gly Gly His Ile Glu Glu Cys Ser Cys Tyr Pro Asn Glu Gly Lys Val Glu Cys Val Cys Arg Asp Asn Trp Thr Gly Thr Asn Arg Pro
                                                                                                                       300

                      950                                                       1000
ATT CTG GTA ATA TCT CCT GAT CTA TCG TAC ACA GTC GGA TAT TTG TGT GCT GGC ATT CCC ACT GAC ACT CCT AGG GGA GAG GAT AGT CAA
Ile Leu Val Ile Ser Pro Asp Leu Ser Tyr Thr Val Gly Tyr Leu Cys Ala Gly Ile Pro Thr Asp Thr Pro Arg Gly Glu Asp Ser Gln
                                                                                                                       330

                      1050                                                      1100
TTC ACA GGC TCA TGC ACA AGC CCT TTG GGA AAT AAA GGA TAC GGT GTA AAG GGC TTC GGG TTT CGA CAA GGA AAT GAC GTA TGG GCC GGA
Phe Thr Gly Ser Cys Thr Ser Pro Leu Gly Asn Lys Gly Tyr Gly Val Lys Gly Phe Gly Phe Arg Gln Gly Asn Asp Val Trp Ala Gly
                                                                                                                       360

                      1150                                                      1200
AGG ACA ATT AGT AGG ACT TCG AGA TCA GGA TTC GAA ATA ATA AAA ATC AGG AAT GGT TGG ACA CAG AAC AGT AAA GAC CAA ATC AGA AAG
Arg Thr Ile Ser Arg Thr Ser Arg Ser Gly Phe Glu Ile Ile Lys Ile Arg Asn Gly Trp Thr Gln Asn Ser Lys Asp Gln Ile Arg Lys
                                                                                                                       390

                      1250
CAA GTG ATT ATT GAT AAC CTA AAT TGG TCA GGA TAT AGC GGT TCT TTC ACA TTG CCG GTT GAA CTA ACA AAA AAA GGA TGT TTA GTC CCC
Gln Val Ile Ile Asp Asn Leu Asn Trp Ser Gly Tyr Ser Gly Ser Phe Thr Leu Pro Val Glu Leu Thr Lys Lys Gly Cys Leu Val Pro
                                                                                                                       420

            1300                                                      1350
TGT TTC TGG GTT GAA ATG ATC AGA GGT AAA CCT GAA GAC ACA ACA ATA TGG ACC TCT AGC AGC TCC ATT GTG ATG TGT GGA GTA GAC CAT
Cys Phe Trp Val Glu MET Ile Arg Gly Lys Pro Glu Asp Thr Thr Ile Trp Thr Ser Ser Ser Ser Ile Val MET Cys Gly Val Asp His
                                                                                                                       450

            1400                                                      1450
AAA ATT GCC AGT TGG TCA TGG CAC GAT GGA GCT ATT CTT CCC TTT GAC ATC GAT AAG ATG TAA tttacgaa aaaaaaaac cccccccccc
Lys Ile Ala Ser Trp Ser Trp His Asp Gly Ala Ile Leu Pro Phe Asp Ile Asp Lys Met
                                                                                    470

    1481
cccccctgcag
```
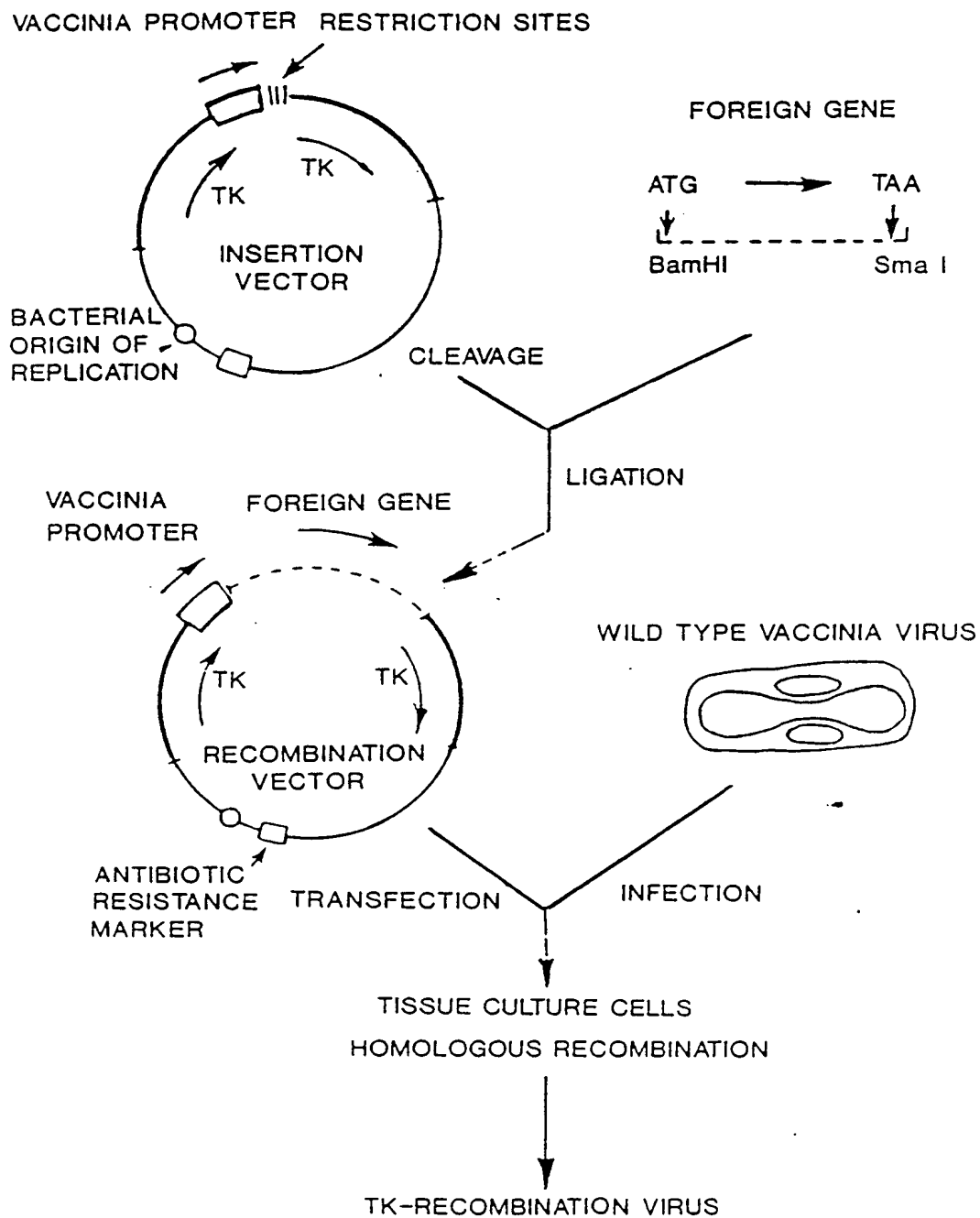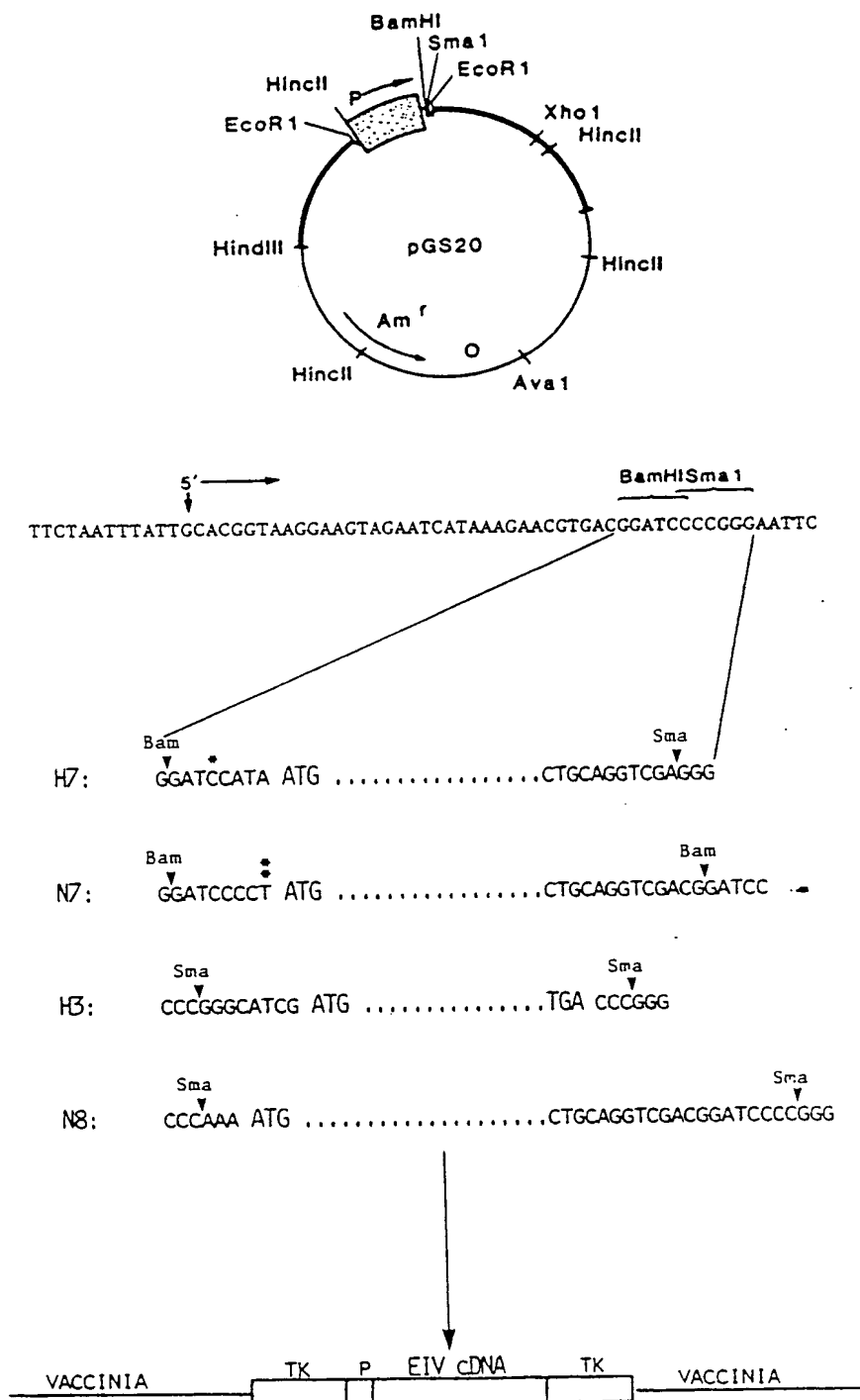
FIG. 4

VACCINIA PROMOTER   RESTRICTION SITES

FOREIGN GENE

ATG ⟶ TAA

BamHI   Sma I

INSERTION VECTOR

BACTERIAL ORIGIN OF REPLICATION

TK   TK

CLEAVAGE

LIGATION

VACCINIA PROMOTER   FOREIGN GENE

RECOMBINATION VECTOR

TK   TK

WILD TYPE VACCINIA VIRUS

ANTIBIOTIC RESISTANCE MARKER   TRANSFECTION   INFECTION

TISSUE CULTURE CELLS

HOMOLOGOUS RECOMBINATION

TK-RECOMBINATION VIRUS

FIG. 5

FIG. 6

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL.   536/27; 424/88; 530/324

INT. CL. -4- CO7H 15/12; A61K 39/00; CO7K 7/10

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
US	536/27; 424/88; 530/324

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	US,A, 4,357,421 Published 2, November 1982, (Emtage)	1-3,5,6, 8-10
A	US,A, 4,455,142 Published 19, June 1984, (Martins)	2,3,5,6,8
X,P	US,A, 4,537,769 Published 27, August 1985, (Cerini)	2,3,5,6,8
X,P	US,A, 4,548,814 Published 22, October 1985, (Rinehart, Jr.)	1-3,5,6, 8-10
A	US,A, 4,474,757 Published 2, October 1984, (Arnon)	2,3,5,6,8
A,P	US,A, 4,578,269 Published 25 March 1986, (Morein)	2,3,5,6,8
A	Ann. Rev. Microbiol. Published 1984, Vol. 3, pages 221-235, (Brown)	1-10

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ³
22 August 1986	0 2 SEP. 1986

International Searching Authority ¹	Signature of Authorized Officer ¹⁰
ISA/US	_Peter K. Plily_

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
A	Journal of American Veterinarian Medical Association, Vol. 143, No. 6 pages 587-590, (Waddell), Published 15 Sept. 1963	1-10
A	Acta Virlogica, Vol. 2, pages 52-61, published 1958, (Sovinova)	1-10
A	Journal of Virology, Published March 1984, pages 857-864, Vol. 49, No. 3, (Mackett)	1-10
A	Proceeding National Academy of Science USA, Vol. 80, pages 7155-7159, Published Dec. 1983, (Smith)	1-10
A	Nature, Vol. 311, Published Sept. 1984, pages 65-69, (Bennink)	1-3,5,6, 8-10
A	Biotechniques, Published Nov./Dec. 1984, pages 306-312, (Smith)	2,3,5,6, 8

Form PCT ISA 210 (extra sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers . . . . . . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers . . . . because they relate to parts of the international application that do not comply with the prescribed require-ments to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I.   Claims 1,9 and 10 drawn to DNA sequences classified in U.S. Class 536.

II. Claims 2,3,5,6 and 8 drawn to compositions and methods of vaccination classified in U.S. Class 424 Subclass 88.

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (May 1986)

III.   Claims 4 and 7 drawn to peptides classified in U.S.
Class 530 Subclass 324.